HUMAN N-TYPE CALCIUM CHANNEL ISOFORM AND USES THEREOF

Related Applications

This application is a divisional of U.S. application serial number 09/268,163, filed March 12, 1999, now pending, and claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application serial number 60/077,901, filed March 13, 1998, the disclosures of which are incorporated by reference herein.

Field of the Invention

The invention pertains to human N-type calcium channel α_{1B} subunit isoforms.

Background of the Invention

Voltage gated calcium channels, also known as voltage dependent calcium channels (VDCCs) are multisubunit membrane spanning proteins which permit controlled calcium influx from an extracellular environment into the interior of a cell. Several types of voltage gated calcium channel have been described in different tissues, including N-type, P/Q-type, L-type and T-type channels. A voltage gated calcium channel permits entry into the cell of calcium upon depolarization of the membrane of the cell, which is a lessening of the difference in electrical potential between the outside and the inside of the cell.

A voltage gated calcium channel contains several proteins, including α_1, α_2, β, and γ subunits. Subtypes of the calcium channel subunits also are known. For instance, α_1 subtypes include α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E} and α_{1S}. Each subunit may have one or more isoforms which result from alternative splicing of RNA in the formation of a completed messenger RNA which encodes the subunit. For example, at least four isoforms of the rat N-type α_{1B} subunit are known (see, e.g., Lin et al., *Neuron* 18:153-166, 1997).

Isoforms of calcium channel α_1 subunits may be expressed differently in different tissues (see, e.g., Lin et al., 1997). Differential expression of subunits isoforms raises the possibility of developing therapeutics which are specific for distinct isoforms of the α_1 subunits, thereby lessening side effects resulting from the use of therapeutics which are effective for more than one calcium channel isoform. Two isoforms of the human N-type calcium channel α_{1B} subunit were published by Williams et al in 1992 (*Science* 257:389-395). Given the existence of several additional rat isoforms in a highly conserved gene family, it is

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surprising that additional human isoforms of the N-type calcium channel α_{1B} subunit have not been discovered. Such isoforms would be useful for developing isoform-specific therapeutics.

Summary of the Invention

The invention provides isolated nucleic acid molecules, unique fragments of those molecules, expression vectors containing the foregoing, and host cells transfected with those molecules. The invention also provides isolated polypeptides and inhibitors of the foregoing nucleic acids and polypeptides which reduce voltage-gated calcium influx. The foregoing can be used in the diagnosis or treatment of conditions characterized by increased or decreased human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity and can be used in methods in which it is therapeutically useful to increase or decrease human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity such as treatments for stroke, pain (e.g., neuropathic pain), traumatic brain injury and conditions characterized by increased or decreased voltage regulated calcium influx. Here, we present the identification of a novel human N-type calcium channel α_{1B} subunit, $h\alpha_{1B+SFVG}$, which plays a role in voltage-gated calcium influx.

It was discovered that a brain α_{1B} calcium channel subunit isoform (splice variant) contains a four amino acid insert relative to published human α_{1B} calcium channel isoforms (SEQ ID NO:5 [GenBank accession number M94172], SEQ ID NO:7 [GenBank accession number M94173]). Surprisingly, this insert, SFVG (SEQ ID NO:2, encoded by SEQ ID NO:1), is similar but not identical to an insert found in a rat α_{1B} channel (GenBank accession number M92905). A significant proportion of the human N-type calcium channel α_{1B} subunit mRNA in brain was found to be the $h\alpha_{1B+SFVG}$ sub-type; given the abundance of its expression the isolation of this sub-type so long after the identification of other α_{1B} isoforms is unexpected. The SFVG-containing human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit also lacks an amino acid sequence, ET, which is present in published human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit isoforms (amino acids 1557-1558 of SEQ ID NOs:5 and 7).

The invention involves in one aspect an isolated human N-type calcium channel α_{1B} subunit polypeptide which includes the amino acid sequence of SEQ ID NO:2 (an $h\alpha_{1B+SFVG}$ polypeptide). In one embodiment, the polypeptide comprises the amino acid sequence of SEQ ID NO:4, and preferably consists of the amino acid sequence of SEQ ID NO:4. In another embodiment the $h\alpha_{1B+SFVG}$ calcium channel polypeptide is a fragment or variant of the foregoing polypeptides, wherein the fragment or variant includes the amino acid sequence of SEQ ID

NO:2 or additions, deletions or substitutions thereof which confer the same function as SEQ ID NO: 2. Preferred variants include those having additions, substitutions or deletions relative to the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide sequence disclosed herein, particularly those variants which retain one or more of the activities of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit, including subunits with or without the ET exon sequence.

According to another aspect of the invention, an isolated nucleic acid molecule which encodes any of the foregoing human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide is provided. In certain embodiments, the nucleic acid molecule includes SEQ ID NO:1. In one preferred embodiment, the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides is encoded by a nucleic acid molecule which comprises the nucleotide sequence of SEQ ID NO:3 (Williams et al. sequence +SFVG, -ET), and which preferably consists of the nucleotide sequence of SEQ ID NO:3. In another embodiment the nucleic acid is an allele of the nucleic acid sequence of SEQ ID NO:3.

In another aspect the invention is an expression vector comprising the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid molecule operably linked to a promoter. Also included within the invention is a host cell transformed or transfected with the expression vector.

According to another aspect of the invention, an agent which selectively binds the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide or a nucleic acid that encodes the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide is provided. By "selectively binds" it is meant that the agent binds the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide or nucleic acid, or any fragment thereof which retains the amino acids of SEQ ID NO:2 or the nucleotides of SEQ ID NO:1, to a greater extent than the agent binds other human N-type calcium channel α_{1B} subunit isoforms, and preferably does not bind other human N-type calcium channel α_{1B} subunit isoforms. In one embodiment, the agent is a polypeptide which binds selectively to the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide. The polypeptide can be a monoclonal antibody, a polyclonal antibody, or an antibody fragment selected from the group consisting of a Fab fragment, a $F(ab)_2$ fragment and a fragment including a CDR3 region. In another embodiment, the agent is an antisense nucleic acid which selectively binds to a nucleic acid encoding the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide. Preferably the foregoing agents are inhibitors (antagonists) or agonists of the calcium channel activity of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit

polypeptide.

According to another aspect of the inventions, a dominant negative human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide is provided. The dominant negative polypeptide is an inhibitor of the function of the calcium channel.

The invention also provides compositions including any of the foregoing polypeptides, nucleic acids or agents in combination with a pharmaceutically acceptable carrier.

In another aspect of the invention a method for inhibiting human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity in a mammalian cell is provided. The method involves the step of contacting the mammalian cell with an amount of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit inhibitor effective to inhibit calcium influx in the mammalian cell. Preferably the inhibitor is selected from the group consisting of a peptide or an antibody which selectively binds the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide, an antisense nucleic acid which binds a nucleic acid encoding human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide and a dominant negative human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide.

According to still another aspect the invention, a method for treating a subject having a stroke, pain (e.g., neuropathic pain), or traumatic brain injury is provided. The method involves the step of administering to a subject in need of such treatment an inhibitor of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide in an amount effective to inhibit voltage regulated calcium influx. In another embodiment of the foregoing methods, the inhibitor is administered prophylactically to a subject at risk of having a stroke.

The human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides and nucleic acids which encode such polypeptides are useful for increasing the amount of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides in a cell. Increasing the amount of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides in a cell results in increased voltage regulated calcium influx. This is useful where it is desired to increase the amount of voltage regulated calcium influx which is mediated by a human N-type calcium channel.

Thus according to another aspect of the invention, a method for increasing human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit expression in a cell is provided. The method involves the step of contacting the cell with a molecule selected from the group consisting of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid and a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide in an amount effective to increase voltage regulated calcium

influx in the cell. In certain embodiments, the cell is contacted with one or more human N-type calcium channel non-h$\alpha_{1B+SFVG}$ subunits, such as a β subunit, or nucleic acids encoding such non-h$\alpha_{1B+SFVG}$ subunits.

According to another aspect of the invention, a method for increasing calcium channel voltage regulated calcium influx in a subject is provided. The method involves the step of administering to a subject in need of such treatment a molecule selected from the group consisting of a human N-type calcium channel h$\alpha_{1B+SFVG}$ subunit nucleic acid and a human N-type calcium channel h$\alpha_{1B+SFVG}$ subunit polypeptide in an amount effective to increase voltage regulated calcium influx in the subject.

According to a further aspect of the invention, a method for identifying lead compounds for a pharmacological agent useful in the treatment of disease associated with increased or decreased voltage regulated calcium influx mediated by a human N-type calcium channel is provided. A cell or other membrane-encapsulated space comprising a human N-type calcium channel h$\alpha_{1B+SFVG}$ subunit polypeptide is provided. The cell or other membrane-encapsulated space preferably is loaded with a calcium-sensitive compound which is detectable in the presence of calcium. The cell or other membrane-encapsulated space is contacted with a candidate pharmacological agent under conditions which, in the absence of the candidate pharmacological agent, cause a first amount of voltage regulated calcium influx into the cell or other membrane-encapsulated space. A test amount of voltage regulated calcium influx then is determined. For example, in a preferred embodiment, fluorescence of a calcium-sensitive compound then is detected as a measure of the voltage regulated calcium influx. If the test amount of voltage regulated calcium influx is less than the first amount, then the candidate pharmacological agent is a lead compound for a pharmacological agent which reduces voltage regulated calcium influx. If the test amount of voltage regulated calcium influx is greater than the first amount, then the candidate pharmacological agent is a lead compound for a pharmacological agent which increases voltage regulated calcium influx.

In another aspect of the invention, methods for identifying compounds which selectively or preferentially bind a human N-type calcium channel h$\alpha_{1B+SFVG}$ subunit isoform are provided. In one embodiment, the method includes providing a first cell or membrane encapsulated space which expresses a human N-type calcium channel h$\alpha_{1B+SFVG}$ subunit isoform, and providing a second cell or membrane encapsulated space which expresses a human N-type calcium channel non-h$\alpha_{1B+SFVG}$ subunit isoform, wherein the second cell or

membrane encapsulated space is identical to the first cell except for the α_{1B} isoform expressed. The first cell or membrane encapsulated space and the second cell or membrane encapsulated space are contacted with a compound, and the binding of the compound to the first cell or membrane encapsulated space and the second cell or membrane encapsulated space is determined. A compound which binds the first cell or membrane encapsulated space but does not bind the second cell or membrane encapsulated space is a compound which selectively binds the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit isoform. A compound which binds the first cell or membrane encapsulated space in an amount greater than the compound binds the second cell or membrane encapsulated space is a compound which preferentially binds the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit isoform. In another embodiment of the method, a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit isoform polypeptide or nucleic acid and a human N-type calcium channel non-$h\alpha_{1B+SFVG}$ subunit isoform polypeptide or nucleic acid are provided and contacted with a compound. The binding of the compound to the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit isoform polypeptide or nucleic acid and the human N-type calcium channel non-$h\alpha_{1B+SFVG}$ subunit isoform polypeptide or nucleic acid then is determined. A compound which binds the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit isoform polypeptide or nucleic acid but does not bind the human N-type calcium channel non-$h\alpha_{1B+SFVG}$ subunit isoform polypeptide or nucleic acid is a compound which selectively binds the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit isoform polypeptide or nucleic acid. A compound which binds the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit isoform polypeptide or nucleic acid in an amount greater than the human N-type calcium channel non-$h\alpha_{1B+SFVG}$ subunit isoform polypeptide or nucleic acid is a compound which preferentially binds the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit isoform polypeptide or nucleic acid. Also included in the invention are compounds identified using the foregoing methods.

According to another aspect of the invention, a method for selectively treating a subject having a condition characterized by aberrant brain neuronal calcium current is provided. The method includes the step of administering to a subject in need of such treatment a pharmacological agent which is selective for a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit, in an amount effective to normalize the aberrant neuronal calcium current. Aberrant means a level of calcium current (calcium influx) which is outside of a normal range as understood in the medical arts. Normalize means that the calcium current is brought within the normal range.

Also presented herein is an identification of characteristics of certain calcium channel subunit isoforms with respect to voltage-dependent activation. It has been discovered, surprisingly, that the presence or absence of an exon comprising the amino acids ET is important for the kinetics of channel activation. Thus, in still other aspects of the invention, a variety of novel assays, screens, recombinant products, model systems (such as animal models) and methods are provided which utilize the unexpected different activation functions between and among the calcium channel subunit isoforms for the identification of novel agents, treatments, etc. useful in the modulation of conditions which arise from or manifest differences in action potential neurotransmitter release, voltage-dependent calcium channel activation, and so on. For example, methods for the identification of agents which alter activation potential dependent neurotransmitter release are provided. The methods include selecting an agent which binds a calcium channel isoform having or lacking a IVS3-S4 ET exon as described herein, and determining calcium channel activation or activation potential dependent neurotransmitter release in the presence and the absence of the agent. In some embodiments, candidate compounds may be screened by such methods. The methods also can include measurement of these parameters in other calcium channel subunits which manifest such differences in activation kinetics, including subunits in which an NP exon is added or is substituted for the ET exon.

Use of the foregoing compositions in the preparation of a medicament, and particularly in the preparation of a medicament for the treatment of stroke, pain (e.g., neuropathic pain), traumatic brain injury, or a condition which results from excessive or insufficient voltage regulated calcium influx, is provided.

These and other aspects of the invention are described in greater detail below.

Brief Description of the Figures

Fig. 1 shows that the presence of ET in domain IVS3-S4 of α_{1B} slows the rate of N-type Ca channel activation.

Fig. 2 shows the impact of alternative splicing in the S3-S4 linkers of the α_{1B} subunit on action potential-dependent Ca influx in a model neuron.

Fig. 3 shows the results of a functional analysis of site-directed mutagenesis of ET splice site in domain IVS3-S4 of the α_{1B} subunit.

Brief Description of the Sequences

SEQ ID NO:1 is the nucleotide sequence of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit cDNA IIIS3-S4 "SFVG" site.

SEQ ID NO:2 is the amino acid sequence of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide IIIS3-S4 "SFVG" site.

SEQ ID NO:3 is the nucleotide sequence of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit cDNA.

SEQ ID NO:4 is the amino acid sequence of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide.

SEQ ID NO:5 is the nucleotide sequence of the coding region of a human α_{1B} calcium channel which lacks the IIIS3-S4 "SFVG" site (Prior art, GenBank accession number M94172).

SEQ ID NO:6 is the amino acid sequence of a human α_{1B} calcium channel which lacks the IIIS3-S4 "SFVG" site (Prior art, GenBank accession number M94172).

SEQ ID NO:7 is the nucleotide sequence of the coding region of a human α_{1B} calcium channel which lacks the IIIS3-S4 "SFVG" site (Prior art, GenBank accession number M94173).

SEQ ID NO:8 is the amino acid sequence of a human α_{1B} calcium channel which lacks the IIIS3-S4 "SFVG" site (Prior art, GenBank accession number M94173).

SEQ ID NO:9 is the nucleotide sequence of the coding region of a rat α_{1B} calcium channel which contains a SFMG site (Prior art, GenBank accession number M92905).

SEQ ID NO:10 is the amino acid sequence of a rat α_{1B} calcium channel which contains a SFMG site (Prior art, GenBank accession number M92905).

SEQ ID NO:11 is the amino acid sequence of an ω-conotoxin peptide from *C. geographus*.

SEQ ID NO:12 is the amino acid sequence of ω-conotoxin peptide from *C. magus*.

SEQ ID NOS:13-28 are primers for PCR and/or sequencing.

Detailed Description of the Invention

The present invention in one aspect involves the identification of a cDNA encoding a novel human isoform of the N-type calcium channel, referred to herein as the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit. As used herein, $h\alpha_{1B+SFVG}$ refers to any human N-type calcium channel α_{1B} subunit clone that contains the SFVG sequence set forth in SEQ ID NO:2. The nucleotide sequence of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit insert, the

IIIS3-S4 "SFVG" site, is presented as SEQ ID NO:1, and the amino acid sequence of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit insert, the IIIS3-S4 "SFVG" site, is presented as SEQ ID NO:2. The 12 nucleotides of SEQ ID NO:1 are inserted immediately following nt 3855 in the coding sequence of the human N-type calcium channel α_{1B} subunit in the codon encoding amino acid 1237, such that the four amino acids of SEQ ID NO:2 are inserted in the polypeptide after amino acid 1237 (see SEQ ID NO:3). The closely related human N-type calcium channel α_{1B-b} subunit, which does not contain the IIIS3-S4 "SFVG" site, was deposited in GenBank under accession numbers M94172 and M94173 (SEQ ID NOs:5 - 8). A related rat N-type calcium channel α_{1B} subunit was deposited in GenBank under accession number M92905 (SEQ ID NOs:9 and 10). Surprisingly, the amino acid sequence of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit differs from the rat amino acid sequence in the SFVG site, which sequence is located in an area of the molecule in which the human and rat amino acid sequences are otherwise 100% identical. This species difference in the very highly conserved protein domain of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit is entirely unexpected, and permits the screening of compounds which selectively bind to and/or modulate the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit. Because the present human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit is a splice variant of other human N-type calcium channel α_{1B} subunits, it is apparent that the invention is meant to embrace human N-type calcium channel α_{1B} subunit variants which vary by alternative splicing of sequences other than the SFVG (SEQ ID NO:2) insert. For example, the invention embraces polypeptides which contain or do not contain an Ala residue immediately following amino acid position 414 of SEQ ID NO:3, or a Glu-Thr insert (ET in single letter code) at amino acid positions 1557-1558 (see, e.g., SEQ ID NO:6), as well as nucleic acid molecules encoding such splice variant polypeptides. As shown in the Examples, the $h\alpha_{1B+SFVG}$ subunit is a significant portion of the α_{1B} calcium channel expressed in human brain, and is differentially distributed in different parts of the brain. This opens the possibility for the selective treatment of disorders which involve those parts of the brain.

The invention involves in one aspect human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids and polypeptides, as well as therapeutics relating thereto. The invention also embraces isolated functionally equivalent variants, useful analogs and fragments of the foregoing nucleic acids and polypeptides; complements of the foregoing nucleic acids; and molecules which selectively bind the foregoing nucleic acids and polypeptides.

The human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids and polypeptides of

the invention are isolated. The term "isolated", as used herein in reference to a nucleic acid molecule, means a nucleic acid sequence: (i) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) synthesized by, for example, chemical synthesis; (iii) recombinantly produced by cloning; or (iv) purified, as by cleavage and electrophoretic or chromatographic separation. The term "isolated", as used herein in reference to a polypeptide, means a polypeptide encoded by an isolated nucleic acid sequence, as well as polypeptides synthesized by, for example, chemical synthetic methods, and polypeptides separated from biological materials, and then purified, using conventional protein analytical or preparatory procedures, to an extent that permits them to be used according to the methods described herein.

As used herein a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid refers to an isolated nucleic acid molecule which codes for a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit. Human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids are those nucleic acid molecules which code for human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides which include the sequence of SEQ ID NO:2. The nucleic acid molecules include the nucleotide sequence of SEQ ID NO:1 and nucleotide sequences which differ from the sequence of SEQ ID NO:1 in codon sequence due to the degeneracy of the genetic code. The human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids of the invention also include alleles of the foregoing nucleic acids, as well as fragments of the foregoing nucleic acids, provided that the allele or fragment encodes the amino acid sequence of SEQ ID NO:2. Such fragments can be used, for example, as probes in hybridization assays and as primers in a polymerase chain reaction (PCR). Preferred human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids include the nucleic acid sequence of SEQ ID NO: 1. Complements of the foregoing nucleic acids also are embraced by the invention.

As used herein "human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity" refers to an ability of a molecule to modulate voltage regulated calcium influx. A molecule which inhibits human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity (an antagonist) is one which inhibits voltage regulated calcium influx via this calcium channel and a molecule which increases human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity (an agonist) is one which increases voltage regulated calcium influx via this calcium channel. Changes in human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity can be measured by changes in voltage regulated calcium influx by *in vitro* assays such as those disclosed herein, including patch-clamp assays and assays employing calcium sensitive fluorescent compounds such as fura-2.

Alleles of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids of the invention can be identified by conventional techniques. For example, alleles of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit can be isolated by hybridizing a probe which includes SEQ ID NO:1 under stringent conditions with a cDNA library and selecting positive clones. Thus, an aspect of the invention is those nucleic acid sequences which code for human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides and which hybridize to a nucleic acid molecule consisting of SEQ ID NO:1 under stringent conditions. The term "stringent conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. More specifically, stringent conditions, as used herein, refers, for example, to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 2.5mM NaH_2PO_4(pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M sodium chloride/0.015M sodium citrate, pH7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetracetic acid. After hybridization, the membrane upon which the DNA is transferred is washed at 2 x SSC at room temperature and then at 0.1 x SSC/0.1 x SDS at temperatures up to 65°C.

There are other conditions, reagents, and so forth which can be used, which result in a similar degree of stringency. The skilled artisan will be familiar with such conditions, and thus they are not given here. It will be understood, however, that the skilled artisan will be able to manipulate the conditions in a manner to permit the clear identification of alleles of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids of the invention. The skilled artisan also is familiar with the methodology for screening cells and libraries for expression of such molecules which then are routinely isolated, followed by isolation of the pertinent nucleic acid molecule and sequencing.

In screening for human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids, a Southern blot may be performed using the foregoing stringent conditions, together with a radioactive probe. After washing the membrane to which the DNA is finally transferred, the membrane can be placed against X-ray film to detect the radioactive signal.

The human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids of the invention also include degenerate nucleic acids which include alternative codons to those present in the native

materials. For example, serine residues are encoded by the codons TCA, AGT, TCC, TCG, TCT and AGC. Each of the six codons is equivalent for the purposes of encoding a serine residue. Thus, it will be apparent to one of ordinary skill in the art that any of the serine-encoding nucleotide triplets may be employed to direct the protein synthesis apparatus, *in vitro* or *in vivo*, to incorporate a serine residue into an elongating human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide. Similarly, nucleotide sequence triplets which encode other amino acid residues include, but are not limited to: CCA, CCC, CCG and CCT (proline codons); CGA, CGC, CGG, CGT, AGA and AGG (arginine codons); ACA, ACC, ACG and ACT (threonine codons); AAC and AAT (asparagine codons); and ATA, ATC and ATT (isoleucine codons). Other amino acid residues may be encoded similarly by multiple nucleotide sequences. Thus, the invention embraces degenerate nucleic acids that differ from the biologically isolated nucleic acids in codon sequence due to the degeneracy of the genetic code.

The invention also provides isolated fragments of SEQ ID NO:3 which include the nucleotide sequence of SEQ ID NO:1. The fragments can be used as probes in Southern blot assays to identify such nucleic acids, or can be used in amplification assays such as those employing PCR. Smaller fragments are those comprising 12, 13, 14, 15, 16, 17, 18, 20, 22, 25, 30, 40, 50, or 75 nucleotides, and every integer therebetween and are useful e.g. as primers for nucleic acid amplification procedures. As known to those skilled in the art, larger probes such as 200, 250, 300, 400 or more nucleotides are preferred for certain uses such as Southern blots, while smaller fragments will be preferred for uses such as PCR. Fragments also can be used to produce fusion proteins for generating antibodies or determining binding of the polypeptide fragments. Likewise, fragments can be employed to produce non-fused fragments of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides, useful, for example, in the preparation of antibodies, in immunoassays, and the like. The foregoing nucleic acid fragments further can be used as antisense molecules to inhibit the expression of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids and polypeptides, particularly for therapeutic purposes as described in greater detail below.

The invention also includes functionally equivalent variants of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit, which include variant nucleic acids and polypeptide which retain one or more of the functional properties of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit, but always including SEQ ID NO:2. For example, variants include a fusion protein

which includes the extracellular and transmembrane domains of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit (including SEQ ID NO:2), which retains the ability to bind ligand and/or transduce a voltage gated calcium current. Still other functionally equivalent variants include variants of SEQ ID NO: 2 which retain functions of subunit including SEQ ID NO: 2. Functionally equivalent variants also include a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit which has had a portion of the extracellular domain (but not SEQ ID NO:2) removed or replaced by a similar domain from another calcium channel α_1 subunit (e.g. a "domain-swapping" variant). Other functionally equivalent variants will be known to one of ordinary skill in the art, as will methods for preparing such variants. The activity of a functionally equivalent variant can be determined using the methods provided herein, in Lin et al., *Neuron* 18:153-166, 1997, and in US patent 5,429,921. Such variants are useful, *inter alia*, in assays for identification of compounds which bind and/or regulate the calcium influx function of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit, and for determining the portions of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit which are required for calcium influx activity.

Variants which are non-functional also can be prepared as described above. Such variants are useful, for example, as negative controls in experiments testing subunit activity, and as inhibition of N-type calcium channel activity.

A human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid, in one embodiment, is operably linked to a gene expression sequence which directs the expression of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid within a eukaryotic or prokaryotic cell. The "gene expression sequence" is any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid to which it is operably linked. The gene expression sequence may, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPRT), adenosine deaminase, pyruvate kinase, β-actin promoter and other constitutive promoters. Exemplary viral promoters which function constitutively in eukaryotic cells include, for example, promoters from the simian virus, papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus, the long terminal repeats (LTR) of Moloney murine leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to

those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters. Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein promoter is induced to promote transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

In general, the gene expression sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid. The gene expression sequences optionally includes enhancer sequences or upstream activator sequences as desired.

The human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid sequence and the gene expression sequence are said to be "operably linked" when they are covalently linked in such a way as to place the transcription and/or translation of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit coding sequence under the influence or control of the gene expression sequence. If it is desired that the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit sequence be translated into a functional protein, two DNA sequences are said to be operably linked if induction of a promoter in the 5' gene expression sequence results in the transcription of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be operably linked to a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid sequence if the gene expression sequence were capable of effecting transcription of that human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid and the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide (including the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit inhibitors described below) of the invention can be delivered to the eukaryotic or prokaryotic cell alone or in association with a vector. In its broadest sense, a

"vector" is any vehicle capable of facilitating: (1) delivery of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid or polypeptide to a target cell or (2) uptake of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid or polypeptide by a target cell. Preferably, the vectors transport the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid or polypeptide into the target cell with reduced degradation relative to the extent of degradation that would result in the absence of the vector. Optionally, a "targeting ligand" can be attached to the vector to selectively deliver the vector to a cell which expresses on its surface the cognate receptor (e.g. a receptor, an antigen recognized by an antibody) for the targeting ligand. In this manner, the vector (containing a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid or a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide) can be selectively delivered to a specific cell. In general, the vectors useful in the invention are divided into two classes: biological vectors and chemical/physical vectors. Biological vectors are more useful for delivery/uptake of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids to/by a target cell. Chemical/physical vectors are more useful for delivery/uptake of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids or human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit proteins to/by a target cell.

Biological vectors include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the nucleic acid sequences of the invention, and free nucleic acid fragments which can be attached to the nucleic acid sequences of the invention. Viral vectors are a preferred type of biological vector and include, but are not limited to, nucleic acid sequences from the following viruses: retroviruses, such as Moloney murine leukemia virus; Harvey murine sarcoma virus; murine mammary tumor virus; Rous sarcoma virus; adenovirus; adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; and polio virus. One can readily employ other vectors not named but known in the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. In general, the retroviruses are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral

expression vectors have general utility for the high-efficiency transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., *"Gene Transfer and Expression, A Laboratory Manual,"* W.H. Freeman C.O., New York (1990) and Murry, E.J. Ed. *"Methods in Molecular Biology,"* vol. 7, Humana Press, Inc., Clifton, New Jersey (1991).

Another preferred virus for certain applications is the adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus can be engineered to be replication-deficient and is capable of infecting a wide range of cell types and species. It further has advantages, such as heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Expression vectors containing all the necessary elements for expression are commercially available and known to those skilled in the art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. Cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA) encoding a human N-type calcium channel h$\alpha_{1B+SFVG}$ subunit polypeptide or fragment or variant thereof. That heterologous DNA (RNA) is placed under operable control of transcriptional elements to permit the expression of the heterologous DNA in the host cell.

Preferred systems for mRNA expression in mammalian cells are those such as pRc/CMV (available from Invitrogen, Carlsbad, CA) that contain a selectable marker such as a gene that confers G418 resistance (which facilitates the selection of stably transfected cell lines) and the human cytomegalovirus (CMV) enhancer-promoter sequences. Additionally, suitable for expression in primate or canine cell lines is the pCEP4 vector (Invitrogen), which contains

an Epstein Barr virus (EBV) origin of replication, facilitating the maintenance of plasmid as a multicopy extrachromosomal element. Another expression vector is the pEF-BOS plasmid containing the promoter of polypeptide Elongation Factor 1α, which stimulates efficiently transcription *in vitro*. The plasmid is described by Mishizuma and Nagata (*Nuc. Acids Res.* 18:5322, 1990), and its use in transfection experiments is disclosed by, for example, Demoulin (*Mol. Cell. Biol.* 16:4710-4716, 1996). Still another preferred expression vector is an adenovirus, described by Stratford-Perricaudet, which is defective for E1 and E3 proteins (*J. Clin. Invest.* 90:626-630, 1992).

In addition to the biological vectors, chemical/physical vectors may be used to deliver a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid or polypeptide to a target cell and facilitate uptake thereby. As used herein, a "chemical/physical vector" refers to a natural or synthetic molecule, other than those derived from bacteriological or viral sources, capable of delivering the isolated human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid or polypeptide to a cell.

A preferred chemical/physical vector of the invention is a colloidal dispersion system. Colloidal dispersion systems include lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system of the invention is a liposome. Liposomes are artificial membrane vesicles which are useful as a delivery vector *in vivo* or *in vitro*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2 - 4.0 μ can encapsulate large macromolecules. RNA, DNA, and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., *Trends Biochem. Sci.*, v. 6, p. 77 (1981)). In order for a liposome to be an efficient nucleic acid transfer vector, one or more of the following characteristics should be present: (1) encapsulation of the nucleic acid of interest at high efficiency with retention of biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information.

Liposomes may be targeted to a particular tissue by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein. Ligands which may be useful for targeting a liposome to a particular cell will depend on the particular cell or tissue type. Additionally when the vector encapsulates a nucleic acid, the vector may be coupled to a nuclear targeting peptide, which will direct the human N-type calcium channel $h\alpha_{1B+SFVG}$

subunit nucleic acid to the nucleus of the host cell.

Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTIN™ and LIPOFECTACE™, which are formed of cationic lipids such as N-[1-(2, 3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in the art and have been described in many publications. Liposomes also have been reviewed by Gregoriadis, G. in *Trends in Biotechnology*, V. 3, p. 235-241 (1985).

Other exemplary compositions that can be used to facilitate uptake by a target cell of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids include calcium phosphate and other chemical mediators of intracellular transport, microinjection compositions, electroporation and homologous recombination compositions (e.g., for integrating a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid into a preselected location within a target cell chromosome).

The invention also embraces so-called expression kits, which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of the previously discussed coding sequences. Other components may be added, as desired, as long as the previously mentioned sequences, which are required, are included.

It will also be recognized that the invention embraces the use of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit cDNA sequences in expression vectors, as well as to transfect host cells and cell lines, be these prokaryotic (e.g., *E. coli*), or eukaryotic (e.g., COS cells, yeast expression systems and recombinant baculovirus expression in insect cells). Especially useful are mammalian cells such as human, pig, goat, primate, etc. They may be of a wide variety of tissue types, and include primary cells and cell lines. Specific examples include neuronal cells including PC12 cells, *Xenopus* oocytes, bone marrow stem cells and embryonic stem cells. The expression vectors require that the pertinent sequence, i.e., those nucleic acids described *supra*, be operably linked to a promoter.

The invention also provides isolated human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides which include the amino acid sequence of SEQ ID NO:2, encoded by the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids described above. The preferred human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide has the amino acid sequence of SEQ ID NO:4. Human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides also embrace alleles, functionally equivalent variants and analogs (those non-allelic polypeptides which vary in

amino acid sequence from SEQ ID NO:4 by 1, 2, 3, 4, 5, or more amino acids) provided that such polypeptides include the amino acids of SEQ ID NO:2 and retain human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity, and fragments of SEQ ID NO:4 which include SEQ ID NO:2. Non-functional variants also are embraced by the invention; these are useful as antagonists of calcium channel function, as negative controls in assays, and the like. Such alleles, variants, analogs and fragments are useful, for example, alone or as fusion proteins for a variety of purposes such as to generate antibodies, or as a component of an immunoassay.

Fragments of a polypeptide preferably are those fragments which retain a distinct functional capability of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide, in particular voltage regulated calcium influx. Other functional capabilities which can be retained in a fragment of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide include interaction with antibodies and interaction with other polypeptides (such as other subunits of the human N-type calcium channel). Those skilled in the art are well versed in methods for selecting fragments which retain a functional capability of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit. Confirmation of the functional capability of the fragment can be carried out by synthesis of the fragment and testing of the capability according to standard methods. For example, to test the voltage regulated calcium influx of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit fragment, one inserts or expresses the fragment in a cell in which calcium influx can be measured. Such methods, which are standard in the art, are described further in the examples.

The invention embraces variants of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides described above. As used herein, a "variant" of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide is a polypeptide which contains one or more modifications to the primary amino acid sequence of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide. Modifications which create a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit variant can be made to a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide for a variety of reasons, including 1) to reduce or eliminate an activity of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide, such as voltage gated calcium influx; 2) to enhance a property of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide, such as protein stability in an expression system or the stability of protein-protein binding; 3) to provide a novel activity or property to a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide, such as addition of an antigenic epitope or addition of a detectable moiety; or 4) to establish

that an amino acid substitution does or does not affect voltage gated calcium influx..

Modifications to a human $h\alpha_{1B+SFVG}$ calcium channel polypeptide are typically made to the nucleic acid which encodes the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids or non-amino acid moieties. Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety, such as biotin, addition of a fatty acid, and the like. Modifications also embrace fusion proteins comprising all or part of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit amino acid sequence, but always including SEQ ID NO:2. One of skill in the art will be familiar with methods for predicting the effect on protein conformation of a change in protein sequence, and can thus "design" a variant human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit according to known methods. One example of such a method is described by Dahiyat and Mayo in *Science* 278:82-87, 1997, whereby proteins can be designed *de novo*. The method can be applied to a known protein to vary a only a portion of the polypeptide sequence. By applying the computational methods of Dahiyat and Mayo, specific variants of a cancer associated antigen polypeptide can be proposed and tested to determine whether the variant retains a desired conformation.

Variants include human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides which are modified specifically to alter a feature of the polypeptide unrelated to its physiological activity. For example, cysteine residues can be substituted or deleted to prevent unwanted disulfide linkages. Similarly, certain amino acids can be changed to enhance expression of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide by eliminating proteolysis by proteases in an expression system (e.g., dibasic amino acid residues in yeast expression systems in which KEX2 protease activity is present).

Mutations of a nucleic acid which encode a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide preferably preserve the amino acid reading frame of the coding sequence, and preferably do not create regions in the nucleic acid which are likely to hybridize to form secondary structures, such as hairpins or loops, which can be deleterious to expression of the variant polypeptide.

Mutations can be made by selecting an amino acid substitution, or by random mutagenesis of a selected site in a nucleic acid which encodes the polypeptide. Variant polypeptides are then expressed and tested for one or more activities to determine which

mutation provides a variant polypeptide with a desired property. Further mutations can be made to variants (or to non-variant human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides) which are silent as to the amino acid sequence of the polypeptide, but which provide preferred codons for translation in a particular host. The preferred codons for translation of a nucleic acid in, e.g., *E. coli*, are well known to those of ordinary skill in the art. Still other mutations can be made to the noncoding sequences of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit gene or cDNA clone to enhance expression of the polypeptide.

The activity of variants of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides can be tested by cloning the gene encoding the variant human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the variant human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide, and testing for a functional capability of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides as disclosed herein. For example, the variant human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide can be tested for ability to provide voltage regulated calcium influx, as set forth below in the examples. Preparation of other variant polypeptides may favor testing of other activities, as will be known to one of ordinary skill in the art.

The skilled artisan will also realize that conservative amino acid substitutions may be made in human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides to provide functionally equivalent variants of the foregoing polypeptides, i.e., variants which retain the functional capabilities of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution which does not alter the relative charge or size characteristics of the polypeptide in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Exemplary functionally equivalent variants of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides include conservative amino acid substitutions of SEQ ID NO:4, but excluding the portion of the polypeptide consisting of SEQ ID NO:2 (SFVG). Conservative substitutions of amino acids include substitutions made amongst amino

acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

Conservative amino-acid substitutions in the amino acid sequence of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide to produce functionally equivalent variants of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides typically are made by alteration of the nucleic acid sequence encoding human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides (e.g., SEQ ID NO:3). Such substitutions can be made by a variety of methods known to one of ordinary skill in the art. For example, amino acid substitutions may be made by PCR-directed mutation, site-directed mutagenesis according to the method of Kunkel (Kunkel, *Proc. Nat. Acad. Sci. U.S.A.* 82: 488-492, 1985), or by chemical synthesis of a gene encoding a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide. Where amino acid substitutions are made to a small unique fragment of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide, such as a leucine zipper domain, the substitutions can be made by directly synthesizing the peptide. The activity of functionally equivalent fragments of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides can be tested by cloning the gene encoding the altered human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the altered human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide, and testing for the ability of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide to transduce voltage regulated calcium influx. Peptides which are chemically synthesized can be tested directly for function.

A variety of methodologies well-known to the skilled practitioner can be utilized to obtain isolated human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit molecules. The polypeptide may be purified from cells which naturally produce the polypeptide by chromatographic means or immunological recognition. Alternatively, an expression vector may be introduced into cells to cause production of the polypeptide. In another method, mRNA transcripts may be microinjected or otherwise introduced into cells to cause production of the encoded polypeptide. Translation of mRNA in cell-free extracts such as the reticulocyte lysate system also may be used to produce polypeptide. Those skilled in the art also can readily follow known methods for isolating human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides. These include, but are not limited to, immunochromatography, HPLC, size-exclusion chromatography, ion-exchange chromatography and immune-affinity chromatography.

The invention as described herein has a number of uses, some of which are described elsewhere herein. For example, the invention permits isolation of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide molecules containing the amino acid sequence of SEQ ID NO:2 by e.g., expression of a recombinant nucleic acid to produce large quantities of polypeptide which may be isolated using standard protocols. As another example, the isolation of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit gene makes it possible for the artisan to diagnose a disorder characterized by loss of expression of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit. These methods involve determining expression of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid, and/or human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides derived therefrom. In the former situation, such determinations can be carried out via any standard nucleic acid determination assay, including the polymerase chain reaction, or assaying with labeled hybridization probes.

The invention also embraces agents which bind selectively to the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit (having or encoding SEQ ID NO:2) and agents which bind preferentially to the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit (having or encoding SEQ ID NO:2) as well as agents which bind to variants and fragments of the polypeptides and nucleic acids as described herein. Selective binding means that the agent binds to the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit but not to human N-type calcium channel non-$h\alpha_{1B+SFVG}$ subunits (i.e., those subunits which do not have or encode SEQ ID NO:2). Preferential binding means that the agent binds more to the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit than to human N-type calcium channel non-$h\alpha_{1B+SFVG}$ subunit, e.g., the agent binds with greater affinity or avidity to the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit having or encoding SEQ ID NO:2. The agents include polypeptides which bind to human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit, and antisense nucleic acids, both of which are described in greater detail below. The agents can inhibit or increase human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity (antagonists and agonists, respectively).

Some of the agents are inhibitors. A human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit inhibitor is an agent that inhibits human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit mediated voltage gated calcium influx. Human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit inhibitors also include dominant negative peptides and known N-type calcium channel inhibitors including the ω-conotoxin peptides and derivative thereof such as ziconotide (SNX-111). Small organic molecule calcium channel inhibitors, such as fluspirilene, NNC

09-0026(-)-trans-1-butyl-4-(4-dimethylaminophenyl)-3- [(4-trifluoromethyl-phenoxy) methyl] piperidinedihydrochloride); SB 201823-A (4-[2-(3,4-dichlorophenoxy)ethyl]-1-pentyl piperidinehydrochloride); NS 649 (2-amino-1-(2,5-dimethoxyphenyl)-5-trifluoromethyl benzimidazole); CNS 1237 (N-acenaphthyl-N'-4-methoxynaphth-1-yl guanidine) and riluzole may also exhibit specificity for the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit.

Calcium influx assays can be performed to screen and/or determine whether a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit inhibitor has the ability to inhibit human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity, and whether the inhibition is selective. As used herein, "inhibit" refers to inhibiting by at least 10% voltage gated calcium influx, preferably inhibiting by at least 25% voltage gated calcium influx, and more preferably inhibiting by at least 40% voltage gated calcium influx as measured by any of the methods well known in the art. An exemplary assay of voltage gated calcium influx is described below in the Examples.

Inhibitors may selectively inhibit $h\alpha_{1B+SFVG}$ based on the state of depolarization of the membrane with which the $h\alpha_{1B+SFVG}$ is associated. It is well known that certain compounds preferentially bind to voltage-gated calcium channels at particular voltages. For example, dihydropyridine compounds preferentially bind to L-type voltage-gated calcium channels when the membrane is depolarized. Bean (*Proc. Nat'l. Acad. Sci.* 81:6388, 1984) described the binding of nitrendipine to cardiac L-type channels only when the membrane is depolarized. Similar results have been found for nimodipine action in sensory neurons (McCarthy & TanPiengco, *J. Neurosci.* 12:2225, 1992).

Activators of human N-type calcium channel $h\alpha_{1B+SFVG}$ activity also are enhanced by the invention. Activators may be identified and/or tested using methods described above for inhibitors. The SFVG site is located in a portion of the $h\alpha_{1B+SFVG}$ channel which is important for voltage dependent gating of Ca^{2+} influx. Therefore, in screening for modulators of $h\alpha_{1B+SFVG}$, including inhibitors and activators (i.e. antagonists and agonists), it is preferred that compounds (e.g. libraries of potential channel inhibitors) are tested for modulation of $h\alpha_{1B+SFVG}$ activity at a variety of voltages which cause partial or complete membrane depolarization, or hyperpolarization. These assays are conducted according to standard procedures of testing calcium channel function (e.g. patch clamping, fluorescent Ca^{2+} influx assays) which require no more than routine experimentation. Using such methods, modulators of $h\alpha_{1B+SFVG}$ activity which are active at particular voltages (e.g. complete membrane depolarization) can be identified. Such compounds are useful for selectively modulating calcium channel activity in

conditions which may display voltage dependence. For example, following a stroke membranes are depolarized and such compounds may be active in selectively blocking calcium channel activity for treatment of stroke. Other uses will be apparent to one of ordinary skill in the art.

In one embodiment the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit inhibitor is an antisense oligonucleotide that selectively binds to a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid molecule, to reduce the expression of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit in a cell. This is desirable in virtually any medical condition wherein a reduction of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity is desirable, e.g., voltage gated calcium influx.

As used herein, the term "antisense oligonucleotide" or "antisense" describes an oligonucleotide that is an oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an mRNA transcript of that gene and, thereby, inhibits the transcription of that gene and/or the translation of that mRNA. The antisense molecules are designed so as to interfere with transcription or translation of a target gene upon hybridization with the target gene or transcript. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence. It is preferred that the antisense oligonucleotide be constructed and arranged so as to bind selectively with the target under physiological conditions, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions. Based upon SEQ ID NO:1, or upon allelic or homologous genomic and/or cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention. In order to be sufficiently selective and potent for inhibition, such antisense oligonucleotides should comprise at least 10 and, more preferably, at least 15 consecutive bases which are complementary to the target, although in certain cases modified oligonucleotides as short as 7 bases in length have been used successfully as antisense oligonucleotides (Wagner et al., *Nature Biotechnol.* 14:840-844, 1996). Most preferably, the antisense oligonucleotides comprise a complementary sequence of 20-30 bases. Although oligonucleotides may be chosen which are antisense to any region of

the gene or mRNA transcripts, in preferred embodiments the antisense oligonucleotides correspond to N-terminal or 5' upstream sites such as translation initiation, transcription initiation or promoter sites. In addition, 3'-untranslated regions may be targeted. Targeting to mRNA splicing sites has also been used in the art but may be less preferred if alternative mRNA splicing occurs. In addition, the antisense is targeted, preferably, to sites in which mRNA secondary structure is not expected (see, e.g., Sainio et al., *Cell Mol. Neurobiol.* 14(5):439-457, 1994) and at which polypeptides are not expected to bind. Thus, the present invention also provides for antisense oligonucleotides which are complementary to allelic or homologous cDNAs and genomic DNAs corresponding to human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid containing SEQ ID NO:1.

In one set of embodiments, the antisense oligonucleotides of the invention may be composed of "natural" deoxyribonucleotides, ribonucleotides, or any combination thereof. That is, the 5' end of one native nucleotide and the 3' end of another native nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleoside linkage. These oligonucleotides may be prepared by art recognized methods which may be carried out manually or by an automated synthesizer. They also may be produced recombinantly by vectors.

In preferred embodiments, however, the antisense oligonucleotides of the invention also may include "modified" oligonucleotides. That is, the oligonucleotides may be modified in a number of ways which do not prevent them from hybridizing to their target but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term "modified oligonucleotide" as used herein describes an oligonucleotide in which (1) at least two of its nucleotides are covalently linked via a synthetic internucleoside linkage (i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred synthetic internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, carboxymethyl esters and peptides.

The term "modified oligonucleotide" also encompasses oligonucleotides with a covalently modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having backbone sugars which are covalently attached to low molecular

weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-alkylated ribose group. In addition, modified oligonucleotides may include sugars such as arabinose instead of ribose. The present invention, thus, contemplates pharmaceutical preparations containing modified antisense molecules that are complementary to and hybridizable with, under physiological conditions, nucleic acids encoding human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides, together with pharmaceutically acceptable carriers.

Antisense oligonucleotides may be administered as part of a pharmaceutical composition. Such a pharmaceutical composition may include the antisense oligonucleotides in combination with any standard pharmaceutically acceptable carriers which are known in the art. The compositions should be sterile and contain a therapeutically effective amount of the antisense oligonucleotides in a unit of weight or volume suitable for administration to a patient. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The characteristics of the carrier will depend on the route of administration. Pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

Agents which bind human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit also include binding peptides which bind to the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit and complexes containing the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit. When the binding polypeptides are inhibitors, the polypeptides bind to and inhibit the activity of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit. To determine whether a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit binding peptide binds to human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit any known binding assay may be employed. For example, the peptide may be immobilized on a surface and then contacted with a labeled human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit. The amount of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit which interacts with the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit binding peptide or the amount which does not bind to the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit binding peptide may then be quantitated to determine whether the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit binding peptide binds to human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit. Further, the binding of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit and a human N-type calcium channel non-$h\alpha_{1B+SFVG}$ subunit can be compared to determine if the binding peptide binds selectively or

preferentially.

The human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit binding peptides include peptides of numerous size and type that bind selectively or preferentially to human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides, and complexes of both human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides and their binding partners. These peptides may be derived from a variety of sources. For example, binding peptides include known N-type calcium channel inhibitors such as the ω-conotoxin peptides GVIA SEQ ID NO: 11 (from *C. geographus*) and MVIIA SEQ ID NO: 12 (from *C. magus*). Other such human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit binding peptides can be provided by modifying the foregoing peptides or by screening degenerate peptide libraries which can be readily prepared in solution, in immobilized form or as phage display libraries. Combinatorial libraries also can be synthesized of peptides containing one or more amino acids. Libraries further can be synthesized of peptoids and non-peptide synthetic moieties.

Phage display can be particularly effective in identifying binding peptides useful according to the invention. Briefly, one prepares a phage library (using e.g. m13, fd, or lambda phage), displaying inserts from 4 to about 80 amino acid residues using conventional procedures. The inserts may represent, for example, a completely degenerate or biased array. One then can select phage-bearing inserts which bind to the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide. This process can be repeated through several cycles of reselection of phage that bind to the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide. Repeated rounds lead to enrichment of phage bearing particular sequences. DNA sequence analysis can be conducted to identify the sequences of the expressed polypeptides. The minimal linear portion of the sequence that binds to the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide can be determined. One can repeat the procedure using a biased library containing inserts containing part or all of the minimal linear portion plus one or more additional degenerate residues upstream or downstream thereof. Yeast two-hybrid screening methods also may be used to identify polypeptides that bind to the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides. Thus, the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides of the invention, or a fragment thereof, can be used to screen peptide libraries, including phage display libraries, to identify and select peptide binding partners of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides of the invention. Such molecules can be used, as described, for screening assays, for purification protocols, for

interfering directly with the functioning of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit and for other purposes that will be apparent to those of ordinary skill in the art.

Peptides may easily be synthesized or produced by recombinant means by those of skill in the art. Using routine procedures known to those of ordinary skill in the art, one can determine whether a peptide which binds to human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit is useful according to the invention by determining whether the peptide is one which inhibits the activity of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit in a voltage gated calcium influx assay, as discussed above.

The human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit binding peptide agent may also be an antibody or a functionally active antibody fragment. Antibodies are well known to those of ordinary skill in the science of immunology. As used herein, the term "antibody" means not only intact antibody molecules but also fragments of antibody molecules retaining human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit binding ability. Such fragments are also well known in the art and are regularly employed both *in vitro* and *in vivo*. In particular, as used herein, the term "antibody" means not only intact immunoglobulin molecules but also the well-known active fragments $F(ab')_2$, and Fab. $F(ab')_2$, and Fab fragments which lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl et al., *J. Nucl. Med.* 24:316-325 (1983)).

In one set of embodiments, the antibody useful according to the methods of the present invention is an intact, fully human anti-human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit monoclonal antibody in an isolated form or in a pharmaceutical preparation. The following is a description of a method for developing a monoclonal antibody that interacts with and inhibits the activity of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit. The description is exemplary and is provided for illustrative purposes only.

Murine monoclonal antibodies may be made by any of the methods known in the art utilizing human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit, or a fragment thereof, as an immunogen provided that the channel or fragment contains the amino acid sequence of SEQ ID NO:2.

Human monoclonal antibodies may be made by any of the methods known in the art, such as those disclosed in US Patent No. 5,567,610, issued to Borrebaeck et al., US Patent No. 5,565,354, issued to Ostberg, US Patent No. 5,571,893, issued to Baker et al, Kozber, *J. Immunol.* 133: 3001 (1984), Brodeur, et al., *Monoclonal Antibody Production Techniques and*

Applications, p. 51-63 (Marcel Dekker, Inc, new York, 1987), and Boerner et al., *J. Immunol.*, 147: 86-95 (1991). In addition to the conventional methods for preparing human monoclonal antibodies, such antibodies may also be prepared by immunizing transgenic animals that are capable of producing human antibodies (e.g., Jakobovits et al., *Proc. Nat'l. Acad. Sci. USA*, 90: 2551 (1993), Jakobovits et al., *Nature*, 362: 255-258 (1993), Bruggermann et al., *Year in Immuno.*, 7:33 (1993) and US Patent No. 5,569,825 issued to Lonberg).

Alternatively the antibody may be a polyclonal antibody specific for human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit which inhibits human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity. The preparation and use of polyclonal antibodies is known to one of ordinary skill in the art.

Significantly, as is well known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W.R. (1986) *The Experimental Foundations of Modern Immunology* Wiley & Sons, Inc., New York; Roitt, I. (1991) *Essential Immunology*, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an $F(ab')_2$ fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are

largely responsible for antibody specificity.

In general, intact antibodies are said to contain "Fc" and "Fab" regions. The Fc regions are involved in complement activation and are not involved in antigen binding. An antibody from which the Fc' region has been enzymatically cleaved, or which has been produced without the Fc' region, designated an "$F(ab')_2$" fragment, retains both of the antigen binding sites of the intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an "Fab'" fragment, retains one of the antigen binding sites of the intact antibody. Fab' fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain, denoted "Fd." The Fd fragments are the major determinants of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity). Isolated Fd fragments retain the ability to specifically bind to antigen epitopes.

The sequences of the antigen-binding Fab' portion of the anti-human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit monoclonal antibodies identified as being useful according to the invention in the assays provided above, as well as the relevant FR and CDR regions, can be determined using amino acid sequencing methods that are routine in the art. It is well established that non-CDR regions of a mammalian antibody may be replaced with corresponding regions of non-specific or hetero-specific antibodies while retaining the epitope specificity of the original antibody. This technique is useful for the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc′ regions to produce a functional antibody. Techniques to humanize antibodies are particularly useful when non-human animal (e.g., murine) antibodies which inhibit human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity are identified. These non-human animal antibodies can be humanized for use in the treatment of a human subject in the methods according to the invention. An example of a method for humanizing a murine antibody is provided in PCT International Publication No. WO 92/04381 which teaches the production and use of humanized murine RSV antibodies in which at least a portion of the murine FR regions have been replaced by FR regions of human origin. Such antibodies, including fragments of intact antibodies with antigen-binding ability, are often referred to as "chimeric" antibodies.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for $F(ab')_2$, and Fab fragments of an anti-human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit monoclonal antibody; chimeric antibodies in which the Fc and/or FR and/or CDR1

and/or CDR2 and/or light chain CDR3 regions of an anti-human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit antibody have been replaced by homologous human or non-human sequences; chimeric $F(ab')_2$ fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions of an anti-human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit antibody have been replaced by homologous human or non-human sequences; and chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences. Thus, those skilled in the art may alter an anti-human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit antibody by the construction of CDR grafted or chimeric antibodies or antibody fragments containing, all or part thereof, of the disclosed heavy and light chain V-region CDR amino acid sequences (Jones et al., *Nature* 321:522, 1986; Verhoeyen et al., *Science* 39:1534, 1988 and Tempest et al., *Bio/Technology* 9:266, 1991), without destroying the specificity of the antibodies for human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit. Such CDR grafted or chimeric antibodies or antibody fragments can be effective in inhibiting human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity in animals (e.g. primates) and humans.

In preferred embodiments, the chimeric antibodies of the invention are fully human monoclonal antibodies. As noted above, such chimeric antibodies may be produced in which some or all of the FR regions of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit have been replaced by other homologous human FR regions. In addition, the Fc portions may be replaced so as to produce IgA or IgM as well as IgG antibodies bearing some or all of the CDRs of the anti-human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit antibody. Of particular importance is the inclusion of the anti-human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit heavy chain CDR3 region and, to a lesser extent, the other CDRs of anti-human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit antibodies. Such fully human chimeric antibodies will have particular utility in that they will not evoke an immune response against the antibody itself.

It is also possible, in accordance with the present invention, to produce chimeric antibodies including non-human sequences. Thus, one may use, for example, murine, ovine, equine, bovine or other mammalian Fc or FR sequences to replace some or all of the Fc or FR regions of the anti-human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit antibody. Some of the CDRs may be replaced as well. Again, however, it is preferred that at least the heavy chain CDR3 region of the anti-human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit antibody be included in such chimeric antibodies and, to a lesser extent, it is also preferred that some or all of the

other CDRs of anti-human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit be included. Such chimeric antibodies bearing non-human immunoglobulin sequences admixed with the CDRs of the human anti-human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit monoclonal antibody are not preferred for use in humans and are particularly not preferred for extended use because they may evoke an immune response against the non-human sequences. They may, of course, be used for brief periods or in immunosuppressed individuals but, again, fully human antibodies are preferred. Because, however, such antibodies may be used for brief periods or in immunosuppressed subjects, chimeric antibodies bearing non-human mammalian Fc and FR sequences but including at least the anti-human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit heavy chain CDR3 are contemplated as alternative embodiments of the present invention.

For inoculation or prophylactic uses, the antibodies of the present invention are preferably intact antibody molecules including the Fc region. Such intact antibodies will have longer half-lives than smaller fragment antibodies (e.g. Fab) and are more suitable for intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, or transdermal administration.

Fab fragments, including chimeric Fab fragments, are preferred in methods in which the antibodies of the invention are administered directly to a local tissue environment. For example, the Fab fragments are preferred when the antibody of the invention is administered directly to the brain. Fabs offer several advantages over $F(ab')_2$ and whole immunoglobulin molecules for this therapeutic modality. First, because Fabs have only one binding site for their cognate antigen, the formation of immune complexes is precluded whereas such complexes can be generated when bivalent $F(ab')_2$ s and whole immunoglobulin molecules encounter their target antigen. This is of some importance because immune complex deposition in tissues can produce adverse inflammatory reactions. Second, because Fabs lack an Fc region they cannot trigger adverse inflammatory reactions that are activated by Fc, such as activation of the complement cascade. Third, the tissue penetration of the small Fab molecule is likely to be much better than that of the larger whole antibody. Fourth, Fabs can be produced easily and inexpensively in bacteria, such as *E. coli*, whereas whole immunoglobulin antibody molecules require mammalian cells for their production in useful amounts. Production of Fabs in *E. coli* makes it possible to produce these antibody fragments in large fermenters which are less expensive than cell culture-derived products.

Smaller antibody fragments and small binding peptides having binding specificity for

the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit which can be used to inhibit human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity also are embraced within the present invention. For example, single-chain antibodies can be constructed in accordance with the methods described in U.S. Patent No. 4,946,778 to Ladner et al. Such single-chain antibodies include the variable regions of the light and heavy chains joined by a flexible linker moiety. Methods for obtaining a single domain antibody ("Fd") which comprises an isolated VH single domain, also have been reported (see, for example, Ward et al., *Nature* 341:644-646 (1989)).

According to the invention human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit inhibitors also include "dominant negative" polypeptides derived from SEQ ID NO:4. A dominant negative polypeptide is an inactive variant of a polypeptide, which, by interacting with the cellular machinery, displaces an active polypeptide from its interaction with the cellular machinery or competes with the active polypeptide, thereby reducing the effect of the active polypeptide. For example, a dominant negative receptor which binds a ligand but does not transmit a signal in response to binding of the ligand can reduce the biological effect of expression of the ligand. Likewise, a dominant negative human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit of an active complex (e.g. N-type calcium channel) can interact with the complex but prevent the activity of the complex (e.g. voltage gated calcium influx).

The end result of the expression of a dominant negative human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide of the invention in a cell is a reduction in voltage gated calcium influx. One of ordinary skill in the art can assess the potential for a dominant negative variant of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide, and using standard mutagenesis techniques to create one or more dominant negative variant polypeptides. For example, given the teachings contained herein of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide, one of ordinary skill in the art can modify the sequence of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide by site-specific mutagenesis, scanning mutagenesis, partial gene deletion or truncation, and the like. See, e.g., U.S. Patent No. 5,580,723 and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. The skilled artisan then can test the population of mutagenized polypeptides for diminution in human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity (e.g., voltage gated calcium influx) and/or for retention of such an activity. Other similar methods for creating and testing dominant negative variants of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide will be apparent to one of ordinary skill in the art.

Each of the compositions of the invention is useful for a variety of therapeutic and non-therapeutic purposes. For example, the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids of the invention are useful as oligonucleotide probes. Such oligonucleotide probes can be used herein to identify genomic or cDNA library clones possessing an identical or substantially similar nucleic acid sequence. A suitable oligonucleotide or set of oligonucleotides, which is capable of hybridizing under stringent hybridization conditions to the desired sequence, a variant or fragment thereof, or an anti-sense complement of such an oligonucleotide or set of oligonucleotides, can be synthesized by means well known in the art (see, for example, *Synthesis and Application of DNA and RNA*, S.A. Narang, ed., 1987, Academic Press, San Diego, CA) and employed as a probe to identify and isolate the desired sequence, variant or fragment thereof by techniques known in the art. Techniques of nucleic acid hybridization and clone identification are disclosed by Sambrook, et al., *Molecular Cloning, A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Plainview, NY (1989), and by Hames, B.D., et al., in *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, DC (1985). To facilitate the detection of a desired nucleic acid sequence, or variant or fragment thereof, whether for cloning purposes or for the mere detection of the presence of the sequence, the above-described probes may be labeled with a detectable group. Such a detectable group may be any material having a detectable physical or chemical property. Such materials have been well-developed in the field of nucleic acid hybridization and, in general, most any label useful in such methods can be applied to the present invention. Particularly useful are radioactive labels. Any radioactive label may be employed which provides for an adequate signal and has a sufficient half-life. If single stranded, the oligonucleotide may be radioactively labeled using kinase reactions. Alternatively, oligonucleotides are also useful as nucleic acid hybridization probes when labeled with a non-radioactive marker such as biotin, an enzyme or a fluorescent group. See, for example, Leary, J.J., et al., *Proc. Natl. Acad. Sci. (USA)* 80:4045 (1983); Renz, M. et al., *Nucl. Acids Res.* 12:3435 (1984); and Renz, M., *EMBO J.* 6:817 (1983).

Additionally, complements of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids can be useful as antisense oligonucleotides, e.g., by delivering the antisense oligonucleotide to an animal to induce a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit "knockout" phenotype. The administration of antisense RNA probes to block gene expression is discussed in Lichtenstein, C., *Nature* 333:801-802 (1988).

Alternatively, the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid of the invention can be used to prepare a non-human transgenic animal. A "transgenic animal" is an animal having cells that contain DNA which has been artificially inserted into a cell, which DNA becomes part of the genome of the animal which develops from that cell. Preferred transgenic animals are primates, mice, rats, cows, pigs, horses, goats, sheep, dogs and cats. Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Charles River (Wilmington, MA), Taconic (Germantown, NY), Harlan Sprague Dawley (Indianapolis, IN), etc. Transgenic animals having a particular property associated with a particular disease can be used to study the affects of a variety of drugs and treatment methods on the disease, and thus serve as genetic models for the study of a number of human diseases. The invention, therefore, contemplates the use of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit knockout and transgenic animals as models for the study of disorders involving voltage gated calcium influx.

A variety of methods are available for the production of transgenic animals associated with this invention. DNA can be injected into the pronucleus of a fertilized egg before fusion of the male and female pronuclei, or injected into the nucleus of an embryonic cell (e.g., the nucleus of a two-cell embryo) following the initiation of cell division. See e.g., Brinster et al., *Proc. Nat. Acad. Sci. USA*, 82: 4438 (1985); Brinster et al., *Cell* 27: 223 (1981); Costantini et al., *Nature* 294: 982 (1981); Harpers et al., *Nature* 293: 540 (1981); Wagner et al., *Proc. Nat. Acad. Sci. USA* 78:5016 (1981); Gordon et al., *Proc. Nat. Acad. Sci. USA* 73: 1260 (1976). The fertilized egg is then implanted into the uterus of the recipient female and allowed to develop into an animal.

An alternative method for producing transgenic animals involves the incorporation of the desired gene sequence into a virus which is capable of affecting the cells of a host animal. See e.g., Elbrecht et al., *Molec. Cell. Biol.* 7: 1276 (1987); Lacey et al., *Nature* 322: 609 (1986); Leopol et al., *Cell* 51: 885 (1987). Embryos can be infected with viruses, especially retroviruses, modified to carry the nucleotide sequences of the invention which encode human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit proteins or sequences which disrupt the native human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit gene to produce a knockout animal.

Another method for producing transgenic animals involves the injection of pluripotent embryonic stem cells into a blastocyst of a developing embryo. Pluripotent stem cells derived from the inner cell mass of the embryo and stabilized in culture can be manipulated in culture

to incorporate nucleotide sequences of the invention. A transgenic animal can be produced from such cells through implantation into a blastocyst that is implanted into a foster mother and allowed to come to term. See e.g., Robertson et al., *Cold Spring Harbor Conference Cell Proliferation* 10: 647 (1983); Bradley et al., *Nature* 309: 255 (1984); Wagner et al., *Cold Spring Harbor Symposium Quantitative Biology* 50: 691 (1985).

The procedures for manipulation of the rodent embryo and for microinjection of DNA into the pronucleus of the zygote are well known to those of ordinary skill in the art (Hogan et al., *supra*). Microinjection procedures for fish, amphibian eggs and birds are detailed in Houdebine and Chourrout, *Experientia*, 47: 897-905 (1991). Other procedures for introduction of DNA into tissues of animals are described in U.S. Patent No., 4,945,050 (Sandford et al., July 30, 1990).

By way of example only, to prepare a transgenic mouse, female mice are induced to superovulate. Females are placed with males, and the mated females are sacrificed by CO_2 asphyxiation or cervical dislocation and embryos are recovered from excised oviducts. Surrounding cumulus cells are removed. Pronuclear embryos are then washed and stored until the time of injection. Randomly cycling adult female mice are paired with vasectomized males. Recipient females are mated at the same time as donor females. Embryos then are transferred surgically. The procedure for generating transgenic rats is similar to that of mice. See Hammer et al., *Cell*, 63:1099-1112 (1990).

Methods for the culturing of embryonic stem (ES) cells and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection also are well known to those of ordinary skill in the art. See, for example, *Teratocarcinomas and Embryonic Stem Cells, A Practical Approach*, E.J. Robertson, ed., IRL Press (1987).

In cases involving random gene integration, a clone containing the sequence(s) of the invention is co-transfected with a gene encoding resistance. Alternatively, the gene encoding neomycin resistance is physically linked to the sequence(s) of the invention. Transfection and isolation of desired clones are carried out by any one of several methods well known to those of ordinary skill in the art (E.J. Robertson, *supra*).

DNA molecules introduced into ES cells can also be integrated into the chromosome through the process of homologous recombination. Capecchi, *Science*, 244: 1288-1292 (1989). Methods for positive selection of the recombination event (e.g., neo resistance) and dual

positive-negative selection (e.g., neo resistance and gancyclovir resistance) and the subsequent identification of the desired clones by PCR have been described by Capecchi, *supra* and Joyner et al., *Nature*, 338: 153-156 (1989). The final phase of the procedure is to inject targeted ES cells into blastocysts and to transfer the blastocysts into pseudopregnant females. The resulting chimeric animals are bred and the offspring are analyzed by Southern blotting to identify individuals that carry the transgene.

Procedures for the production of non-rodent mammals and other animals have been discussed by others. See Houdebine and Chourrout, *supra*; Pursel et al., *Science* 244: 1281-1288 (1989); and Simms et al., *Bio/Technology*, 6: 179-183 (1988).

Inactivation or replacement of the endogenous N-type calcium channel $h\alpha_{1B+SFVG}$ subunit gene can be achieved by a homologous recombination system using embryonic stem cells. The resultant transgenic non-human mammals (preferably primates) having a knockout N-type calcium channel $h\alpha_{1B+SFVG}$ subunit characteristic may be made transgenic for the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit and used as a model for screening compounds as modulators (agonists or antagonists/inhibitors) of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit. In this manner, such therapeutic drugs can be identified.

Additionally, a normal or mutant version of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit can be inserted into the mouse (or the animal) germ line to produce transgenic animals which constitutively or inducibly express the normal or mutant form of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit. These animals are useful in studies to define the role and function of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit in cells. These studies are particularly useful in animals, which do not normally express human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit, such as non-primates.

A human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide, or a fragment thereof, also can be used to isolate human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit native binding partners, including, e.g., the N-type calcium channel. Isolation of such binding partners may be performed according to well-known methods. For example, isolated human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides can be attached to a substrate (e.g., chromatographic media, such as polystyrene beads, or a filter), and then a solution suspected of containing the N-type calcium channel may be applied to the substrate. If a N-type calcium channel which can interact with human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides is present in the solution, then it will bind to the substrate-bound human N-type calcium channel $h\alpha_{1B+SFVG}$

subunit polypeptide. The N-type calcium channel then may be isolated. Other polypeptides which are binding partners for human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit may be isolated by similar methods without undue experimentation.

The compositions of the invention are also useful for therapeutic purposes. Accordingly the invention encompasses a method for inhibiting human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity in a mammalian cell. The invention further provides methods for reducing or increasing human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity in a cell. In one embodiment, the method involves contacting the mammalian cell with an amount of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit inhibitor effective to inhibit voltage gated calcium influx in the mammalian cell. Such methods are useful *in vitro* for altering voltage gated calcium influx for the purpose of, for example, elucidating the mechanisms involved in stroke, pain, e.g., neuropathic pain, and traumatic brain injury and for restoring the voltage gated calcium influx in a cell having a defective human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit. *In vivo*, such methods are useful, for example, for reducing N-type voltage gated calcium influx, e.g., to treat stroke, pain, e.g., neuropathic pain, traumatic brain injury, or any condition in which human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity is elevated.

An amount of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit inhibitor which is effective to inhibit voltage gated calcium influx in the mammalian cell is an amount which is sufficient to reduce voltage gated calcium influx by at least 10%, preferably at least 20%, more preferably 30% and still more preferably 40%. An amount of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit which is effective to increase voltage gated calcium influx in the mammalian cell is an amount which is sufficient to increase voltage gated calcium influx by at least 10%, preferably at least 20%, more preferably 30% and still more preferably 40%. Such alterations in voltage gated calcium influx can be measured by the assays described herein.

As described above with respect to inhibitors, modulators of $h\alpha_{1B+SFVG}$ may selectively inhibit or increase $h\alpha_{1B+SFVG}$ function based on the state of depolarization of the membrane with which the $h\alpha_{1B+SFVG}$ is associated. Therefore, in screening for modulators of $h\alpha_{1B+SFVG}$, it is preferred that compounds (e.g. synthetic combinatorial libraries, natural products, peptide libraries, etc.) are tested for modulation of $h\alpha_{1B+SFVG}$ activity at a variety of voltages which cause partial or complete membrane depolarization, or hyperpolarization. These assays are conducted according to standard procedures of testing calcium channel function (e.g. patch clamping, fluorescent Ca^{2+} influx assays) which require no more than routine experimentation.

Using such methods, modulators of $h\alpha_{1B+SFVG}$ activity which are active at particular voltages (e.g. complete membrane depolarization) can be identified. Such compounds are useful for selectively modulating calcium channel activity in conditions which may display voltage dependence.

The invention also encompasses a method for increasing human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit expression in a cell or subject. It is desirable to increase human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit in a subject that has a disorder characterized by a deficiency in voltage gated calcium influx. The amount of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit can be increased in such cell or subject by contacting the cell with, or administering to the subject, a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acid or a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide of the invention to the subject in an amount effective to increase voltage gated calcium influx in the cell or the subject. An increase in human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit activity can be measured by the assays described herein, e.g., assays of calcium influx.

The invention also contemplates gene therapy. The procedure for performing *ex vivo* gene therapy is outlined in U.S. Patent 5,399,346 and in exhibits submitted in the file history of that patent, all of which are publicly available documents. In general, it involves introduction *in vitro* of a functional copy of a gene into a cell(s) of a subject which contains a defective copy of the gene, and returning the genetically engineered cell(s) to the subject. The functional copy of the gene is under operable control of regulatory elements which permit expression of the gene in the genetically engineered cell(s). Numerous transfection and transduction techniques as well as appropriate expression vectors are well known to those of ordinary skill in the art, some of which are described in PCT application WO95/00654. *In vivo* gene therapy using vectors such as adenovirus, retroviruses, herpes virus, and targeted liposomes also is contemplated according to the invention.

The preparations of the invention are administered in effective amounts. An effective amount is that amount of a pharmaceutical preparation that alone, or together with further doses, produces the desired response. In the case of treating a condition characterized by aberrant voltage gated calcium influx, the desired response is reducing or increasing calcium influx to a level which is within a normal range. Preferably, the change in calcium influx produces a detectable reduction in a physiological function related to the condition, e.g., a reduction in neurotoxicity following stroke. The responses can be monitored by routine

methods. In the case of a condition where an increase in voltage gated calcium influx is desired, an effective amount is that amount necessary to increase said influx in the target tissue. The converse is the case when a reduction in influx is desired. An increase or decrease in neurotransmitter release also could be measured to monitor the response.

Such amounts will depend, of course, on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. It is preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

Generally, doses of active compounds would be from about 0.01 mg/kg per day to 1000 mg/kg per day. It is expected that doses ranging from 50-500 mg/kg will be suitable and in one or several administrations per day. Lower doses will result from other forms of administration, such as intravenous administration. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of compound, although fewer doses typically will be given when compounds are prepared as slow release or sustained release medications.

When administered, the pharmaceutical preparations of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptably compositions. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

The human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit inhibitors or human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids and polypeptides useful according to the invention may be combined, optionally, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The pharmaceutical compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; and phosphoric acid in a salt.

The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular compound selected, the severity of the condition being treated and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, interdermal, or parenteral routes. The term "parenteral" includes subcutaneous, intravenous, intrathecal, intramuscular, or infusion. Intravenous or intramuscular routes are not particularly suitable for long-term therapy and prophylaxis.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a

syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit inhibitor or human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids and polypeptides, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intrathecal, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

Other delivery systems can include time-release, delayed release or sustained release delivery systems such as the biological/chemical vectors is discussed above. Such systems can avoid repeated administrations of the active compound, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. Use of a long-term sustained release implant may be desirable. Long-term release, are used herein, means that the implant is constructed and arranged to delivery therapeutic levels of the active ingredient for at least 30 days, and preferably 60 days. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

The invention further provides efficient methods of identifying pharmacological agents or lead compounds for agents useful in the treatment of conditions associated with aberrant voltage gated cell calcium influx mediated by human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit and the compounds and agents so identified. Generally, the screening methods involve assaying for compounds which inhibit or enhance voltage gated calcium influx through human N-type calcium channels. Such methods are adaptable to automated, high throughput screening of compounds. Examples of such methods are described in US patent 5,429,921.

A variety of assays for pharmacological agents are provided, including, labeled *in vitro*

protein binding assays, Ca^{2+} influx assays, etc. For example, protein binding screens are used to rapidly examine the binding of candidate pharmacological agents to a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit. The candidate pharmacological agents can be derived from, for example, combinatorial peptide libraries. Convenient reagents for such assays are known in the art. An exemplary cell-based assay of calcium influx involves contacting a neuronal cell having a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit with a candidate pharmacological agent under conditions whereby the influx of calcium can be stimulated by application of a voltage to the test system, i.e., by membrane depolarization. Specific conditions are well known in the art and are described in Lin et al., *Neuron* 18:153-166, 1997, and in US patent 5,429,921. A reduction in the voltage gated calcium influx in the presence of the candidate pharmacological agent indicates that the candidate pharmacological agent reduces the induction of calcium influx of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit in response to the voltage stimulus. An increase in the voltage gated calcium influx in the presence of the candidate pharmacological agent indicates that the candidate pharmacological agent increases the induction of calcium influx of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit in response to the voltage stimulus. Methods for determining changes in the intracellular calcium concentration are known in the art and are addressed elsewhere herein.

Human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit used in the methods of the invention can be added to an assay mixture as an isolated polypeptide (where binding of a candidate pharmaceutical agent is to be measured) or as a cell or other membrane-encapsulated space which includes a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide. In the latter assay configuration, the cell or other membrane-encapsulated space can contain the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit as a preloaded polypeptide or as a nucleic acid (e.g. a cell transfected with an expression vector containing a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit). In the assays described herein, the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide can be produced recombinantly, or isolated from biological extracts, but preferably is synthesized *in vitro*. Human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptides encompass chimeric proteins comprising a fusion of a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide with another polypeptide, e.g., a polypeptide capable of providing or enhancing protein-protein binding, or enhancing stability of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide under assay conditions. A polypeptide fused to a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide or fragment thereof may also provide means of

readily detecting the fusion protein, e.g., by immunological recognition or by fluorescent labeling.

The assay mixture also comprises a candidate pharmacological agent. Typically, a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a different response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration of agent or at a concentration of agent below the limits of assay detection. Candidate agents encompass numerous chemical classes, although typically they are organic compounds. Preferably, the candidate pharmacological agents are small organic compounds, i.e., those having a molecular weight of more than 50 yet less than about 2500. Candidate agents comprise functional chemical groups necessary for structural interactions with polypeptides, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups and more preferably at least three of the functional chemical groups. The candidate agents can comprise cyclic carbon or heterocyclic structure and/or aromatic or polyaromatic structures substituted with one or more of the above-identified functional groups. Candidate agents also can be biomolecules such as peptides, saccharides, fatty acids, sterols, isoprenoids, purines, pyrimidines, derivatives or structural analogs of the above, or combinations thereof and the like. Where the agent is a nucleic acid, the agent typically is a DNA or RNA molecule, although modified nucleic acids having non-natural bonds or subunits are also contemplated.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides, synthetic organic combinatorial libraries, phage display libraries of random peptides, and the like. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds can be readily modified through conventional chemical, physical, and biochemical means. Further, known pharmacological agents may be subjected to directed or random chemical modifications such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs of the agents.

Candidate agents can be selected randomly or can be based on existing compounds which bind to and/or modulate the function of N-type calcium channels. For example,

compounds which are known to inhibit N-type calcium channels include fluspirilene, ziconotide (SNX-111), the ω-conotoxin peptides GVIA (SEQ ID NO: 11) and MVIIA (SEQ ID NO: 12), as well as small organic molecule calcium channel inhibitors, such as fluspirilene, NNC 09-0026(-)-trans-1-butyl-4-(4-dimethylaminophenyl)-3-[(4-trifluoromethyl-phenoxy) methyl] piperidinedihydrochloride); SB 201823-A (4-[2-(3,4-dichlorophenoxy)ethyl]-1-pentyl piperidinehydrochloride); NS 649 (2-amino-1-(2,5-dimethoxyphenyl)-5-trifluoromethyl benzimidazole); CNS 1237 (N-acenaphthyl-N'-4-methoxynaphth-1-yl guanidine) and riluzole. Therefore, a source of candidate agents are libraries of molecules based on the foregoing N-type calcium channel inhibitors, in which the structure of the inhibitor is changed at one or more positions of the molecule to contain more or fewer chemical moieties or different chemical moieties. The structural changes made to the molecules in creating the libraries of analog inhibitors can be directed, random, or a combination of both directed and random substitutions and/or additions. One of ordinary skill in the art in the preparation of combinatorial libraries can readily prepare such libraries based on the existing N-type calcium channel inhibitors.

A variety of other reagents also can be included in the mixture. These include reagents such as salts, buffers, neutral proteins (e.g., albumin), detergents, etc. which may be used to facilitate optimal protein-protein and/or protein-nucleic acid binding. Such a reagent may also reduce non-specific or background interactions of the reaction components. Other reagents that improve the efficiency of the assay such as protease inhibitors, nuclease inhibitors, antimicrobial agents, and the like may also be used.

The mixture of the foregoing assay materials is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit transduces a control amount of voltage gated calcium influx. For determining the binding of a candidate pharmaceutical agent to a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit, the mixture is incubated under conditions which permit binding. The order of addition of components, incubation temperature, time of incubation, and other parameters of the assay may be readily determined. Such experimentation merely involves optimization of the assay parameters, not the fundamental composition of the assay. Incubation temperatures typically are between 4°C and 40°C. Incubation times preferably are minimized to facilitate rapid, high throughput screening, and typically are between 1 minute and 10 hours.

After incubation, the level of voltage gated calcium influx or the level of specific

binding between the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide and the candidate pharmaceutical agent is detected by any convenient method available to the user. For cell free binding type assays, a separation step is often used to separate bound from unbound components. The separation step may be accomplished in a variety of ways. Conveniently, at least one of the components is immobilized on a solid substrate, from which the unbound components may be easily separated. The solid substrate can be made of a wide variety of materials and in a wide variety of shapes, e.g., microtiter plate, microbead, dipstick, resin particle, etc. The substrate preferably is chosen to maximize signal to noise ratios, primarily to minimize background binding, as well as for ease of separation and cost.

Separation may be effected for example, by removing a bead or dipstick from a reservoir, emptying or diluting a reservoir such as a microtiter plate well, rinsing a bead, particle, chromatographic column or filter with a wash solution or solvent. The separation step preferably includes multiple rinses or washes. For example, when the solid substrate is a microtiter plate, the wells may be washed several times with a washing solution, which typically includes those components of the incubation mixture that do not participate in specific bindings such as salts, buffer, detergent, non-specific protein, etc. Where the solid substrate is a magnetic bead, the beads may be washed one or more times with a washing solution and isolated using a magnet.

Detection may be effected in any convenient way for cell-based assays such as a calcium influx assay. The calcium influx resulting from voltage stimulus of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide typically alters a directly or indirectly detectable product, e.g., a calcium sensitive molecule such as fura-2-AM. For cell free binding assays, one of the components usually comprises, or is coupled to, a detectable label. A wide variety of labels can be used, such as those that provide direct detection (e.g., radioactivity, luminescence, optical or electron density, etc). or indirect detection (e.g., epitope tag such as the FLAG epitope, enzyme tag such as horseradish peroxidase, etc.). The label may be bound to a human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit polypeptide or the candidate pharmacological agent.

A variety of methods may be used to detect the label, depending on the nature of the label and other assay components. For example, the label may be detected while bound to the solid substrate or subsequent to separation from the solid substrate. Labels may be directly detected through optical or electron density, radioactive emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, streptavidin-biotin conjugates,

etc. Methods for detecting the labels are well known in the art.

The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention and are not to be construed to limit the scope of the invention.

Examples

Example 1: Analysis of human brain N-type calcium channel splice variants

The abundance of splice variants of N-type calcium channels in human brain was determined using polymerase chain reaction analysis and RNase protection assays as described in Lin et al. (*Neuron* 18:153-166, 1997). Human N-type calcium channel α_{1B} subunit clones were sequenced by standard methods of nucleotide sequencing and it was determined that one type of clone had a 12 nucleotide insert (SEQ ID NO:1) as compared to previously published human N-type calcium channel α_{1B} subunit sequences. The present human N-type calcium channel α_{1B} subunit nucleic acid molecule (designated $h\alpha_{1B+SFVG}$) corresponds to the published nucleotide sequence for human N-type calcium channel α_{1B} subunits with the 12 nucleotide insert located after nucleotide 3855 (as numbered in Williams et al., *Science* 257:389-395, 1992). The nucleotide sequence of SEQ ID NO:1 supplies the third base of the codon encoding Ser1237, three new codons (Ser1238, Phe1239 and Val1240), and the first two bases of codon Gly1241, as shown in SEQ ID NO:3: tcG AGC TTC GTG GGa (insert in caps). This insert thus encodes a four amino acid insert in the protein which is similar to, but surprisingly is not identical to, amino acids 1236-1239 of a rat N-type calcium channel α_{1B} subunit (SEQ ID NO:10, GenBank accession number M92905). The human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit was found to make up a significant portion of the N-type calcium channel α_{1B} subunits mRNA in human brain. It was also determined that the $h\alpha_{1B+SFVG}$ subunit was differentially distributed in different parts of the brain, e.g. in certain portions of the brain $h\alpha_{1B+SFVG}$ was more highly expressed than in other portions of the brain.

Example 2: Construction of human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit nucleic acids

The human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit containing the SFVG insert is constructed according to standard procedures described in, e.g., *Current Protocols in Molecular Biology* (F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York), using PCR primers

which contain the nucleotides encoding SFVG (e.g., SEQ ID NO:1) to amplify the published human N-type calcium channel α_{1B} subunit nucleic acid. Fragments generated by PCR are then assembled by ligation to prepare a complete cDNA encoding the human $h\alpha_{1B+SFVG}$ subunit.

Example 3: Function of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit

The voltage gated calcium channel activity of the human N-type calcium channel $h\alpha_{1B+SFVG}$ subunit is tested using to the methods described in Lin et al. (1997) for a rat N-type calcium channel subunit, and as described in Example 4 below.

Example 4: Activation differences in rat N-type calcium channels ± the ET exon

Functional assessment of the calcium channel α_{1B} cDNA constructs

The functional properties of all calcium (Ca) channel α_{1B} cDNA constructs described in this paper were assessed in the *Xenopus* oocyte expression system. All methods and procedures were essentially the same as described in Lin et al. (1997). cRNAs were *in vitro* transcribed using the mMESSAGE mMACHINE kit (Ambion) from the various α_{1B} cDNA constructs subcloned into the *Xenopus* β-globin expression vector (pBSTA; Goldin & Sumikawa et al., *Methods Enzymol.* 207:279-297, 1992). 46 nl of a 750ng/μl cRNA solution was injected into defolliculated oocytes using a precision nanoinjector (Drummond). N-type Ca channel currents were recorded 6-7 days after injection. At least 15 minutes prior to recording, oocytes were injected with 46 nl of a 50 mM solution of BAPTA (1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate). This we have found critical to minimize activation of an endogenous Ca^{2+}-activated Cl^- current, even when Ba^{2+} is the charge carrier (Lin et al., 1997). Cells exhibiting slowly deactivating tail currents, indicative of the presence of Ba^{2+}-dependent activation of the Ca-activated Cl^- current, were excluded from the analysis.

N-type Ca^{2+} channel currents were recorded from oocytes using the two microelectrode voltage-clamp recording technique (Warner amplifier; OC-725b). A virtual ground circuit eliminated the need for series resistance compensation when recording large currents. Micropipettes of 0.8-1.5 MΩ and 0.3-0.5MΩ resistance when filled with 3 M KCl were used for the voltage and current recording electrodes, respectively. Oocytes expressing Ca^{2+} channel currents usually had resting membrane potentials between -40 and -50 mV when impaled with two electrodes. A grounded metal shield was placed between the two electrodes to increase the settling time of the clamp. Recording solutions contained 5 mM $BaCl_2$, 85 mM

tetraethylammonium, 5mM KCl, and 5mM HEPES (pH adjusted to 7.4 with methanesulfonic acid). The recording temperature was between 19°C and 22°C.

The properties of each mutant construct were assessed by expressing it together with appropriate controls (ΔET α_{1B} and +ET α_{1B}). Each mutant was tested in three separate batches of oocytes and within each batch, recordings were made from at least six oocytes for each mutant construct and control. Recordings from the oocytes expressing the various Ca channel α_{1B} constructs were randomized throughout the data collection period.

Data analysis.

Data were acquired on-line and leak subtracted using a P/4 protocol (PClampV6.0; Axon Inst.). Voltage-steps were applied every 10-30 seconds depending on the duration of the step, from a holding potential of -80 mV. Ca channel currents recorded under these conditions showed little run-down over the duration of the recordings. Three sets of current voltage-relationships were obtained from each cell using step depolarizations of 26.3 ms, 650 ms and 2.6 s in duration and digitized at 25 kHz, 10 kHz and 250 Hz, respectively. Exponential curves (activation and inactivation) were fit to the data using curve fitting routines in PClamp (Axon Instr.) and Origin (Microcal). Inactivation time constants in the range of 70-800 msec were estimated from currents evoked by the longest depolarization (2.6 s). Activation time constants were best resolved from currents evoked by the shortest depolarizations (26.3 ms; sampled at 25 kHz).

Modeling Ca entry.

A one-compartment cell model employing standard compartmental modeling techniques in NEURON (Hines & Carnevale, *Neural Comput.* 9:1179-1209, 1997) was used to predict the amount of Ca entering a neuron expressing either rn$\alpha_{1B\text{-}b}$ or rn$\alpha_{1B\text{-}b}$ N-type Ca channel currents. The cell had a total membrane area of 1250 μm^2, 0.75 $\mu F/cm^2$ specific membrane capacitance and 30 $k\Omega cm^2$ specific membrane resistance. For action potential simulation a fast sodium conductance (g_{Na}) and a delayed rectifying potassium conductance ($g_{K,DR}$) were included (Mainen & Sejnowski, *Science* 268:1503-1506, 1995) each with densities of 300 $pS/\mu m^2$. Ca^{2+} influx was mediated by a fast calcium conductance (g_{Ca}; Yamada et al., Multiple channels and calcium dynamics. *In Methods in Neuronal Modeling*, Koch, C. & Segev, I., Eds. pp 97-134, 1989) with a density of 1 $pS/\mu m^2$. Resultant currents were calculated

using conventional Hodgkin-Huxley kinetic schemes according to the formulae given below. The resting membrane potential was set at -70 mV and Na and K current reversal potentials at +50 mV and -75 mV, respectively. The calcium channel was computed using the Goldman-Hodgkin-Katz equation. Extracellular Ca concentration was 2.5 mM and the intracellular Ca concentration computed using entry via I_{Ca} and removal via a first order pump $d[Ca^{2+}]_i/dt=(-1x10^5.I_{Ca}/2F)-([Ca^{2+}]_i - [Ca^{2+}]_\infty)/\tau_R$, where $[Ca^{2+}]_\infty$ = 10 nM and τ_R = 80 ms. The time constants and maximal conductances were developed at room temperature and were therefore scaled to 37°C using a Q_{10} of 2.3. Formulae used for calculation of various currents were as follows:

Sodium current (I_{Na}), $m^3 \cdot h$: $\alpha_{m,\,Na} = 0.182 \cdot (v + 25)/(1-e^{(v+25)/9})$; $\beta_{m,\,Na} = -0.124 \cdot (v+25)/(1-e^{-(v+25)/9})$

$\alpha_{h,\,Na} = 0.024 \cdot (v + 40)/(1-e^{-(v+40)/5})$; $\beta_{h,\,Na} = -0.0091 \cdot (v+65)/(1-e^{-(v+65)/5})$; $h_{\infty,\,Na} = 1/(1-e^{-(v+55/6\,2)})$

Delayed rectifier ($I_{K(DR)}$), m: $\alpha_{m,\,K(DR)} = 0.02 \cdot (v - 25)/(1-e^{-(v+25)/9})$;

$\beta m_{,\,K(DR)} = -0.002 \cdot (v - 25)/(1-e^{(v+25)/9})$

High threshold, N-type calcium current (I_{Ca}), m·h: $m_{\infty,\,Ca} = 1/(1+e^{-(v-3)/8})$;

$\tau_{m,\,Ca} = 7.8/(e^{(v+6)/16})$; $h_{Ca} = K/(K+[Ca^{2+}]_i)$ with K = 0.01 mM.

The brain-dominant form, $rn\alpha_{1B\text{-}d}$, was then modeled by shifting the voltage-dependence of the N-type Ca channel conductance activation variable ($m_{\infty,\,Ca}$) by -7 mV, and decreasing the activation time constant ($\tau_{m,\,Ca}$) by 33% (Lin et al., 1997 and see Fig. 1A).

Ribonuclease protection assay.

The procedures are essentially the same as those described in Lin et al. (1997). Total RNA was purified from various neuronal tissue of adult rats using a guanidium thiocyanate and phenol-chloroform extraction protocol (adapted from Chomczynski & Sacchi, *Anal. Biochem.* 162:156-159, 1987). ^{32}P-labeled antisense RNA probes overlapping ET (nt 4379-4836) in $rn\alpha_{1B\text{-}b}$ and NP (nt4605-4930) in $rb\alpha_{1A}$ (Starr et al., *Proc. Natl. Acad. Sci. USA*, 88:5621-5625, 1991) were constructed from linearized plasmids (pGEM-T vector) containing appropriate RT-PCR-derived sub-clones using the Maxi-script kit (Ambion). Probes were gel purified and stored as ethanol precipitates. 1 μg of RNA purified from sympathetic or sensory ganglia or 5 μg of RNA isolated from various CNS tissues were precipitated with 2 x 10^5 cpm of probe and resuspended in 30 μl hybridization buffer containing: 60% formamide; 0.4 M NaCl; 10 mM EDTA and 40 mM PIPES at pH 6.4. Samples were denatured at 85°C and allowed to hybridize overnight at 60°C. The samples were then digested in a 350 μl reaction mix containing: 0.3 M

NaCl, 5mM EDTA, 3.5 µl of the RNase Cocktail (Ambion) and 10 mM Tris at pH 7.5, then treated with proteinase K, extracted and precipitated with 10 µg of tRNA as carrier. After resuspension in 30 µl formamide loading buffer, the samples wee denatured and separated on a 5% polyacrylamide gel. After exposure to a phosphor imaging plate to quantity relative band intensities (Fuji BAS 1000), the gel was subsequently exposed to film with an intensifying screen for 4-5 days at -80°C.

Site-directed mutagenesis

A recombinant PCR-based technique was used to introduce mutations (QT, EA, AT, AA, NP) at the ET site in the IVS3-S4 linker of $\alpha_{1B\text{-}b}$. A pair of primers 5'-attcttgtggtcatcgccttgag (Bup 3460; SEQ ID NO: 13) and 5'-gacaggcctccaggagcttggtg (Bdw 5623; SEQ ID NO: 14) flanked a region of the clone that contained two restriction sites RsrII (nt3510) and BglII (nt5465) located on either side of ET (nt4674). A second primer pair contained the desired mutation and directly overlapped the ET site (Bdwmut and Bupmut; see below). Two separate PCRs were performed with Bup 3460 and Bdwmut, and Bupmut and Bdw 5623. The PCR product then served as template for a second round of PCR using Bup 3460 and Bdw 5623 generating the final mutant PCR fragment that was subsequently subcloned into $rn\alpha_{1B\text{-}b}$ at the Rsr II and Bgl II sites. Mutants were screened by restriction digest and confirmed by DNA sequencing. All PCR was performed using Expand High Fidelity (Boehringer Mannheim). The mutagenesis primers used were as follows:

ET/AT:	Bupmut 5'-gagattgcgGCAACGaacaacttcatc-3': SEQ ID NO: 15
	Bdwmut 5'-aagttgttCGTTTCcgcaatctccg-3'; SEQ ID NO: 16
ET/QT:	Bupmut 5'-gagattgcgCAGACGaacaacttcatc-3'; SEQ ID NO: 17
	Bdwmut 5'-aagttgttCGTCTGcgcaatctccg-3'; SEQ ID NO: 18
ET/EA:	Bupmut 5'-gagattgcgGAAGCTaacaacttcatc-3'; SEQ ID NO: 19
	Bdwmut 5'-aagttgttAGCTTCcgcaatctccg-3'; SEQ ID NO: 20
ET/AA:	Bupmut 5'-gagattgcgGCAGCTaacaacttcatc-3'; SEQ ID NO: 21
	Bdwmut 5'-aagttgttAGCTGCcgcaatctccg-3'; SEQ ID NO: 22
ET/NP:	Bupmut 5'-gagattgcgAACCCTaacaacttcatc-3'; SEQ ID NO: 23
	Bdwmut 5'-aagttgttAGGGTTcgcaatctccg-3'; SEQ ID NO: 24

Genomic analysis

The IVS3-S4 region of the rat α_{1B} and α_{1A} genes were analyzed by genomic PCR. Primer pairs were directed to the IVS3 and IVS4 membrane spanning regions that were presumed to reside in the 5' and 3' exons flanking the ET and NP insertions of the α_{1B} and α_{1A} genes, respectively. PCR was performed in a 50µl reaction mix containing 250 ng rat liver genomic DNA, 250 µM of each nucleotide and 0.4 µM of each primer. After a pre-incubation for 15 min at 92°C, 0.75 µl enzyme mix was added to start the amplification. The resultant gDNA products were gel purified, cloned into pGEM-T (Promega) and sequenced. The α_{1B} primers generated two bands of ~11 kb and ~900 bases. The 11 kb band was derived from the α_{1B} gene and contained the desired ET encoding exon in IVS3-S4. The 900 base product resulted from amplification of the equivalent site in the α_{1E} gene that contained a relatively short ~700 bp intron and no intervening exon. The α_{1A} primers generated a single 9 kb PCR product that was confirmed to be derived from the α_{1A} gene by DNA sequencing (Yale University sequencing facility). Primers were as follows:

α_{1A}: Aup4737 5'-tgcctggaacatcttcgactttgtga; SEQ ID NO: 25

Adw4876 5'-cagaggagaatgcggatggtgtaacc; SEQ ID NO: 26

α_{1B}: Bup4599 5'-cagagatgcctggaacgtctttgac; SEQ ID NO: 27

Bdw4744 5'-ataacaagatgcggatggtgtagcc; SEQ ID NO: 28

Alternative splicing in the putative S3-S4 extracellular linkers affects channel activation but not inactivation kinetics

In a previous study it was shown that rn$\alpha_{1B\text{-}b}$ (ΔSFMG/+ET) and rn$\alpha_{1B\text{-}d}$ (+SFMG/ΔET) N-type currents differ with respect to their activation kinetics when expressed in *Xenopus* oocytes (compare Δ/+ and +/Δ in Fig. 1A,B; see also Lin et al., 1997). Inactivation kinetics of the two splice variants have not, however, been compared (Lin et al., 1997). In the present study depolarizations of durations of between 26 ms and 2.6 s were employed to permit the resolution of both the time course of Ca channel activation *and* inactivation. Rat N-type calcium channel subunits (rn$\alpha_{1B\text{-}b}$ [Δ/+] and rn$\alpha_{1B\text{-}d}$ [+/Δ]) were expressed in *Xenopus* oocytes and resulting N-type Ca channel currents recorded using 5 mM Ba as the charge carrier (Fig. 1). Fig. 1A shows the averaged, normalized Ca channel current induced by the expression in *Xenopus* oocytes of four different α_{1B} constructs. Currents were evoked by step depolarizations

to 0 mV from a holding potential of -80 mV. Each trace represents the average, normalized current calculated from at least 6 oocytes. SFMG-containing clones are distinguished from SFMG-lacking clones by thin and thick lines and arrows, respectively. Fig. 1B shows a plot of average activation time constants (nat. log) at different test potentials (between -20 and +10 mV) for clones +/+ (□), Δ/+ (•), +/Δ (○) and Δ/Δ (■). The presence of SFMG in domain IIIS3-S4 did not affect the rate of channel activation. There was no significant difference in τ_{activ} between clones +/+ and Δ/+ or between clones +/Δ and Δ/Δ ($p>0.1$ at all potentials between -20 mV and +10 mV). The presence of ET in domain IVS3-S4 slowed channel activation kinetics. τ_{activ} values for clones +/+ and Δ/+ were significantly slower compared to +/Δ and Δ/Δ, at all test potentials between -20 mV and +10 mV ($p<.05$).

N-type Ca channel currents evoked by depolarization to 0 mV or higher, inactivated with a bi-exponential time course (τ_{fast} 100-150 ms and τ_{slow} 700-800 ms). The inactivation time constants of the cloned channels expressed in *Xenopus* oocytes ($rn\alpha_{1B\text{-}b}$, Δ/+ and $rn\alpha_{1B\text{-}d}$, +/Δ) were weakly voltage-dependent consistent with studies of native N-type Ca channels of bullfrog sympathetic neurons (Jones & Marks, 1989). The fast and slow inactivation time constants of $rn\alpha_{1B\text{-}b}$ and $rn\alpha_{1B\text{-}d}$ currents evoked by step depolarizations to between 0 mV and +30 mV were not significantly different. In contrast, the rates of channel activation of the two variants in the same cells were significantly different (Fig. 1A,B). On the basis of these observations it was concluded that alternative splicing in domains IIIS3-S4 and IVS3-S4 of the α_{1B}-subunit altered the time course of N-type Ca channel activation but had no effect on inactivation kinetics. These findings are consistent with the close proximity of the S3-S4 linkers to their respective S4 helices that are the putative voltage sensors of the 6 transmembrane family of voltage-gated ion channels. In contrast, the domains of the Ca channels α_{1B} subunit implicated in voltage-dependent inactivation of N-type Ca channels (IS6 and flanking putative extracellular and intracellular linkers; Zhang et al., *Nature* 372:97-100, 1994) are likely to be more distant from the S3-S4 linker splice sites.

The observed differences in the properties of $rn\alpha_{1B\text{-}b}$ and $rn\alpha_{1B\text{-}d}$ currents are of sufficient magnitude to impact action potential-induced Ca entry.

An assumption that motivates the present study is that the differences in the kinetics and voltage-dependence of activation of $rn\alpha_{1B\text{-}b}$ and $rn\alpha_{1B\text{-}d}$ N-type Ca channel currents are sufficient to influence the magnitude and time course of voltage-dependent calcium entry in

native cells. A direct test of this hypothesis, however, is complicated by the inability to manipulate selectively the expression or activity of individual splice variants in their native environment. To date no isoform-specific pharmacological tools or antibodies to target Ca channel α_{1B} S3-S4 splice variants exist. Therefore, the available information was used to estimate the relative effectiveness of rn$\alpha_{1B\text{-}b}$ and rn$\alpha_{1B\text{-}d}$ N-type currents to support action potential-induced Ca influx in a model neuron (Hines & Carnevale, 1997). A one-compartment model was used to predict the time course and magnitude of calcium entry in a neuron during action potential-induced depolarization. Simulated action potentials with time courses similar to those recorded in native sympathetic neurons (Yamada et al., 1989; Fig. 2A) were used to trigger voltage-dependent Ca influx in model neurons (Na, K and Ca current densities of 300, 300 and 1 pS/μF, respectively) expressing either rn$\alpha_{1B\text{-}b}$ or rn$\alpha_{1B\text{-}d}$ N-type Ca channel currents. A simulated action potential was evoked by a 10 ms, 40 pA current step (Fig. 2A); a comparison of the resultant N-type channel current (Fig. 2B) and time course of intracellular calcium concentration (Fig. 2C) expected in a model neuron expressing either rn$\alpha_{1B\text{-}b}$ (Δ/+; solid line) or rn$\alpha_{1B\text{-}d}$ (+/Δ; dashed line)-type channels is shown. A shift in the voltage-dependence of the N-type Ca channel conductance activation variable ($m_{\infty, Ca}$) by -7 mV, and a decrease in the activation time constrant ($\tau_{m, Ca}$) by 33% expected for rn$\alpha_{1B\text{-}d}$ (Lin et al., 1997; and see Fig. 1A), resulted in a total increase in charge transfer and peak intracellular Ca concentration of 49% and 48%, respectively. A -50% increase in the total charge transfer (Fig. 2B) and peak intracellular Ca concentration (Fig. 2C) is predicted during an action potential in a neuron expressing rn$\alpha_{1B\text{-}d}$-type Ca channels (dashed line) relative to rn$\alpha_{1B\text{-}b}$ (solid line). All other factors being constant, the functional differences between rn$\alpha_{1B\text{-}b}$ and rn$\alpha_{1B\text{-}d}$ N-type Ca channel currents would be expected to significantly impact the amount of calcium that enters a neuron during action potential-dependent excitation.

Splicing of ET in domain IVS3-S4 underlies the major functional difference between rn$\alpha_{1B\text{-}b}$ and rn$\alpha_{1B\text{-}d}$

rn$\alpha_{1B\text{-}b}$ and rn$\alpha_{1B\text{-}d}$ differ in composition by 6 amino acids located in two distinct regions of the Ca channel α_{1B} subunit (SFMG in domain IIIS3-S4 and ET in domain IVS3-S4). To separate the relative contribution of SFMG in domain IIIS3-S4 and ET in domain IVS3-S4 to the different gating kinetics observed between rn$\alpha_{1B\text{-}b}$ (ΔSFMG/+ET) and rn$\alpha_{1B\text{-}d}$ (+SFMG/ΔET) two additional clones, +/+ and Δ/Δ were constructed and the functional

properties of all four clones were compared. Fig. 1 (A and B) demonstrates that the presence of the dipeptide sequence ET in domain IVS3-S4 is directly correlated with the altered activation kinetics of rnα_{1B-b} currents compared to rnα_{1B-d}. Activation time constants measured from N-type Ca channel currents in oocytes expressing clone Δ/+ (rnα_{1B-b}) and +/+ were indistinguishable and 1.5 fold slower on average than those induced by the expression of clones +/Δ (rnα_{1B-d}) and Δ/Δ (Fig. 1A,B). The presence of ET in domain IVS3-S4 also influenced the voltage-dependence of channel activation. A comparison of the mid-points of the rising phase of the peak current-voltage plots ($V_{½}$) generated for the two ET containing clones, Δ/+ (rnα_{1B}; -7.8 ± 0.6 mV, n=6) and +/+ (-9.7 ± 1.0 mV, n=6) shows that they are not significantly different from each other ($p>0.05$, students' t-test). Likewise, $V_{½}$ values estimated from two ET-lacking constructs, +/Δ (rnα_{1B-d}; -15.4 ± 0.4 mV, n=7) and Δ/Δ (-13.4±0.7, n=6), were not significantly different from each other ($p>0.05$) and activated at potentials that were, on average, 6 mV more negative compared to ET-containing clones Δ/+ and +/+. While the presence of ET in domain IVS3-S4 dominates in regulating the voltage-dependence of activation, the analysis does reveal a small contribution of SFMG. SFMG-containing clones (+/Δ and +/+) activated at potentials that were 2 mV hyperpolarized compared to those that lacked SFMG (Δ/+ and Δ/Δ). A 2 mV shift in the voltage-dependence of activation was not significant at the 5 % level, in a comparison of $V_{½}$ values from clones Δ/+ and +/+, but did reach significance in a comparison of +/Δ and Δ/Δ ($p<0.025$, students t-test).

The pattern of expression of ET-containing Ca channel α_{1B} mRNA in different regions of the nervous system

Fig. 1 indicates that alternative splicing of ET within domain IVS3-S4 of the Ca channel α_{1B}- subunit accounts for the major functional differences between rnα_{1B-b} and rnα_{1B-A}. This prompted a systemic analysis of the expression pattern of the six bases in α_{1B} mRNA that encoded ET (gaa acg). It was previously shown that ET-containing α_{1B} (+ET α_{1B}) mRNA was in very low abundance in total rat brain extracts (Lin et al., 1997). To determine whether ET-lacking α_{1B} (ΔET α_{1B}) mRNA dominated throughout the central nervous system RNA isolated from spinal cord, cerebellum, cortex, hippocampus, hypothalamus, medulla and thalamus of adult rats was analyzed by ribonuclease protection assay. In all regions tested >90% of the α_{1B} mRNA expressed in the central nervous system lacked the ET encoding sequence. In contrast, in sympathetic and sensory ganglia the majority of α_{1B} mRNA contained the ET encoding

sequence. Together these findings suggest that +ET α_{1B} subunits are primarily restricted to neurons of the peripheral nervous system. Consistent with this RNA isolated from human brain and trigeminal ganglia was analyzed and analogous patterns of expression were observed: low levels of +ET α_{1B} mRNA in brain and high levels (>90%) in ganglia.

Site-directed mutagenesis within IVS3-S4

Having shown that alternative splicing of the ET encoding sequence in the IVS3-S4 linker of α_{1B} has a significant effect on the kinetics and voltage-dependence of N-type Ca channel gating, the use of site-directed mutagenesis was employed to determine the relative importance of each amino acid, glutamate and threonine. A series of mutants in which ET was replaced with either QT, AT, EA, AA or NP were constructed (Fig. 3) from clone Δ/+ (rn $\alpha_{1B\text{-}b}$) which served as the background structure. The mutant constructs were then expressed in *Xenopus* oocytes and their properties compared to clones +ET (100% slow; Fig. 3) and ΔET (100% fast; Fig. 3). All mutants expressed equally well in the *Xenopus* oocyte expression system.

The role of the glutamate in domain IVS3-S4 was of major interest because it should be negatively charged at neutral pH and consequently might influence the gating machinery of the channel via electrostatic interactions. Fig. 3, however, shows that replacing glutamate with glutamine resulted in a channel that activated only slightly faster than +ET α_{1B} (Fig. 3; QT). Substituting alanine for glutamate (AT) decreased τ_{act} but, consistent with the QT mutant, suggests that the presence of a negative charge in IVS3-S4 (glu) does not underlie the slow gating kinetics of the +ET α_{1B} variant. Similarly, alanine substitution of either threonine alone (EA) or together with glutamate (AA) generated channels with activation kinetics that were intermediate between +ET α_{1B} and ΔET α_{1B} clones. Together, these results suggest that the presence of both glutamate and threonine in the IVS3-S4 linker is necessary to reconstitute the relatively slow channel opening rates characteristic of N-type Ca channel α_{1B}-subunits that dominate in sensory and sympathetic ganglia.

Sequence comparisons of several cDNAs encoding α_1-subunits of other voltage-gated Ca channels suggests that alternative splicing in the IVS3-S4 linker could be a general mechanism for regulating voltage-dependent Ca channel gating. This has recently been demonstrated for α_{1A} (Sutton et al., *Soc. Neurosci. Abs.* 24:21, 1998), a Ca channel subunit that is closely related both structurally and functionally to the N-type Ca channel α_{1B} subunit. A

comparison of the IVS3-S4 region of various mammalian α_{1A} cDNAs derived from kidney, pancreas and brain (see also Yu et al., *Proc. Natl. Acad. Sci. USA* 89:10494-10498, 1992; Ligon et al., *J. Biol. Chem.* 273:13905-13911, 1998; Sutton et al., 1998) is consistent with alternative splicing of six bases encoding Asp Pro (NP) amino acids in this region. The distribution of +NP α_{1A} and ΔNP α_{1A} mRNAs in different regions of the rat nervous system has not been quantified. Therefore RNase protection analysis was used to determine the expression pattern of the IVS3-S4 splice variants of α_{1A}. Low levels of +NP α_{1A} mRNA were found in rat, spinal cord, striatum and thalamus, a pattern that parallels the low levels of +ET α_{1B} mRNA in the CNS. However, the pattern of NP expression in the cerebellum, cortex and hippocampus did not conform to this picture since mRNA isolated from these tissues contained a significant proportion of +NP α_{1A} mRNAs. In fact, in the hippocampus +NP α_{1A} mRNAs dominated (~60%). Consistent with the abundance of +ET α_{1B} mRNAs in peripheral tissue, the majority of α_{1A} mRNA in superior cervical and dorsal root ganglia contained the six bases encoding NP in domain IVS3-S4 of α_{1A}. The absolute level of α_{1A} mRNA expressed in sympathetic neurons was very low as expected from the absence of P-type currents in recordings from rat sympathetic neurons (Mintz et al., 1992).

The high degree of sequence homology between α_{1B} and α_{1A} in the IVS3-S4 linker region together with the finding that a 6 base sequence is alternatively spliced at both these sites, suggested that ET and NP share a common functional role. To test this hypothesis the functional impact on N-type Ca channel currents of replacing ET in rn$\alpha_{1B\text{-}b}$ with NP was studied. Figure 3 shows that the +NP α_{1B} mutant gives rise to N-type Ca channel currents in oocytes with gating kinetics indistinguishable from wild-type (i.e. +ET α_{1B}). Activation time constants were estimated from currents induced by the expression of the various mutant α_{1B} constructs (QT, AT, EA, AA, NP) in oocytes and compared to clones ET and ΔET (A). Shifts in the activation time constants of the mutant channels, relative to clones ET and ΔET (100% slow) and ΔET (100% fast) are plotted (B). Each point represents data collected from at least 18 oocytes per mutant (each mutant was tested in three separate batches of oocytes and within each experiment at least 6 oocytes per mutant were analyzed). Values plotted are means ± standard errors from the three data sets. The asterisk indicates a significant slowing of the activation time constant compared to clone ET ($P<0.05$).

ET is encoded by a six base exon in the IVS3-S4 linker region of the α_{1B} gene

The existence of an alternatively spliced exon in the IVS3-S4 region of the rat Ca channel α_{1B} gene has been hypothesized (Lin et al., 1997), but not yet confirmed. Genomic analysis was therefore undertaken to locate the splice junctions in the IVS3-S4 region of the α_{1B} gene and to pinpoint the precise location of the putative six-base, ET encoding exon. PCR amplification from rat genomic DNA using primers designed to hybridize to the transmembrane spanning S3 and S4 helices flanking IVS3-S4 in α_{1B} revealed the presence of a long ~10 kb stretch of intron sequence. DNA sequencing established the location of exon/intron and intron/exon boundaries and conserved ag-gt splice junction signature sequences immediately 5′ and 3′ to the putative ET insertion site. A six-base cassette exon encoding ET was located 8 kb into the 5′ intron and establishes that ET-α_{1B} variants are generated by alternative splicing. The exon/intron structure in the IVS3-S4 linker region of the closely related rat α_{1A} gene was also determined. The rat α_{1A} gene also contained a long stretch of intron sequence (~8 kb) and ag-gt splice junctions at the 5′ (gt) and 3′ (at) ends of the intronic segment. The precise location of the NP encoding cassette exon in the rat α_{1A} gene has not been determined but conclude that it must reside within the 8 kb of intron sequence in the IVS3-S4 linker region. Tissue-specific alternative splicing of six base cassette exons in the IVS3-S4 linkers of both α_{1A} and α_{1B} explains the presence of splice variants of these subunits in the mammalian brain and underscores the high level of conservation between these two functionally related genes. The genomic structure of the more distantly related rat α_{1E} gene that encodes a pharmacologically and functionally distinct class of Ca channel (Soong et al., *Science* 260:1133-1136, 1993) also was analyzed. The α_{1E} gene contains a ~700 bp intron in the IVS3-S4 linker region and no obvious intervening exon. The absence of an alternatively spliced cassette exon in the IVS3-S4 linker region of the α_{1E} gene is consistent with RNase protection analysis of α_{1E} mRNA from rat brain which revealed no evidence of sequence variations in this IVS3-S4 linker.

Each of the foregoing patents, patent applications and references is hereby incorporated by reference. While the invention has been described with respect to certain embodiments, it should be appreciated that many modifications and changes may be made by those of ordinary skill in the art without departing from the spirit of the invention. It is intended that such modification, changes and equivalents fall within the scope of the following claims.

What is claimed is: